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Clostridium difficile: expression of virulence factors, resistance to disinfectants and interactions with human cells

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Declaration

The author performed all the investigations and procedures presented in this thesis, unless otherwise stated.

Publications

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Abstract

Clostridium difficile is the most common cause of nosocomial diarrhoea today. Through the changing epidemiology of *C. difficile* infection, the emergence and decline of different strains of varying virulence and a broad spectrum of disease from asymptomatic carriage and mild infection to severe pseudomembranous colitis have been observed. The main aim of this three-part thesis was to identify bacterial factors that might explain these variations by comparing five *C. difficile* strains - strain 630, an historic strain, strain VPI 10463, a reference strain, the hypervirulent ribotype 027 and the current locally endemic ribotypes 001 and 106.

The first study focussed on the growth-related phenotypic and genotypic expression of virulence factors in *C. difficile*. Growth was studied over twenty-four hours, with simultaneous assessment of toxin and spore production. Total toxin production was measured by a commercial ELISA, while a quantitative ELISA for toxin A and a quantitative cytotoxicity assay for toxin B were developed for individual toxin levels, and spores were enumerated by viable counts. Ribotype 027 produced large amounts of toxin A and toxin B and was the second highest spore producer after ribotype 106. Growth may not affect virulence, but the ability to produce more toxins and spores could. To study the transcription of the genes involved in these processes, a real-time RT-PCR was developed. The transcription of the pathogenicity locus (*tcdA-E*) that regulates toxin production in *C. difficile*, and of *spo0A*, the initiator of sporulation, was studied. There were three key observations: firstly, the transcription of *tcdC*, the negative regulator of toxin production, did not decrease over time, suggesting it has a modulatory rather than repressive effect on the process. Secondly, *tcdE* expression was highest in ribotype 027, which might explain its hypertoxicity by greater toxin release. Thirdly, there was almost steady state expression of *spo0A* during the exponential growth phase in ribotypes 106 and 027, the highest spore producers, suggesting prolonged activation of sporulation. Thus, distinct inter-strain differences exist between *C. difficile* strains *in vitro*, which could mirror their virulence *in vivo*, and several traits contribute synergistically to the hypervirulence of ribotype 027.

The second study aimed to identify suitable laboratory disinfectants against *C. difficile*. The efficacy of four commonly-used disinfectants and one decontaminant was tested; one disinfectant was a chlorine-based agent commonly used in hospitals. In conventional susceptibility tests, all five agents were effective against vegetative cells and spores of *C. difficile*. However, only the chlorine-based disinfectant was effective against spores dried onto surfaces, but this too required more than two minutes of treatment. The presence of organic matter significantly impaired the efficacy of the non-chlorine agents. The spores of epidemic strains were destroyed less effectively and exposure to sub-MIC levels of disinfectant increased sporulation, especially in ribotype 001, a common outbreak strain. Environmental sampling of the laboratory and surrounding areas showed considerable dissemination of *C. difficile,* highlighting the need for effective decontamination in conjunction with basic hygiene methods like hand-washing.

The third study examined the biological activity of *C. difficile*. Macrophages were challenged *in vitro* with S-layer proteins, flagella, heat-shock proteins and culture supernatants of the five strains and cytokine production was measured by specially developed ELISAs. No significant inter-strain differences were observed, although the epidemic strains generally elicited a slightly greater cytokine response. Using epithelial cell lines it was observed that epidemic strains showed greater adherence; from inhibition assays, flagella and S-layer proteins were found to contribute equally to this. Through these studies, inter-strain differences between epidemic and historic isolates were identified with respect to virulence factors, survival in the environment and possible behaviour within the host. A sum of these observations suggests increased virulence in contemporary versus historical *C. difficile* strains.

Finally, a supplementary study characterising a collection of ribotype 027 strains isolated in Scotland and the Netherlands by typing schemes, gene sequencing, susceptibility testing and phenotypic studies was performed. In agreement with other studies, the clonality of these hypervirulent strains was observed.

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List of abbreviations

AAC	Antibiotic-associated colitis
ADP	Adenosine diphosphate
AFNOR	Association Française de Normalisation
AHP	Accelerated hydrogen peroxide
AIM	Anaerobe identification medium
AOAC	Association of Official Analytical Chemists
APC	Allophycocyanin
AP-PCR	Arbitrarily primed polymerase chain reaction
APS	Ammonium persulphate
AST	Antibiotic susceptibility testing
BHI	Brain heart infusion
BSA	Bovine serum albumin
CA-CDI	Community-associated *Clostridium difficile* infection
CCFA	Cycloserine cefoxitin frustose egg yolk agar
CCEY	Cefoxitin cylcoserine egg yolk
CD	Cluster of differentiation
CDAD	*Clostridium difficile* associated disease
CDI	*Clostridium difficile* infection
cDNA	Complementary deoxyribonucleic acid
CEN	European Committee for Standardisation
CLSI	Clinical and laboratory standards institute
CROP	Combined repetitive oligopeptide
Ct	Cycle threshold
CWP	Cell wall protein
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunoassay
FBS	Foetal bovine serum

FITC	Fluorescein isothiocyante
GALT	Gut-associated lymphoid tissue
GDH	Glutamate dehydrogenase
GDP	Guanosine diphosphate
gDNA	Genomic deoxyribonucleic acidly
GHCl	Guanidine hydrochloride
GI	Gastrointestinal
GTP	Guanosine triphosphate
HA-CDI	Healthcare-associated *Clostridium difficile* infection
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HI	Heat inactivated
HMW	High molecular weight
HRP	Horse radish peroxidase
HSP	Heat-shock protein
Ig	Immunoglobulin
IGM	Initial growth medium
IL	Interleukin
$InsP_6$	Inositol hexakiphosphate
KCl	Potassium chloride
LAL	Limulus amoebocyte lysate
LMW	Low molecular weight
LPS	Lipopolysaccharide
LRW	LAL reagent water
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MLVA	Multilocus variable-number tandem-repeat analysis
MOPS	Morpholinepropanesulfonic acid
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAP	North American pulse-field type
NGM	Normal growth medium
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PaLoc	Pathogenicity locus
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
Pen/Strep	Penicillin-Streptomycin
PFA	Paraformaldehyde
PFGE	Pulsed field gel electrophoresis
PMA	Phorbol 12-myristate 13-acetate
PMC	Pseudomembranous colitis
PPE	Personal protective equipment
PPI	Proton pump inhibitor
PRR	Pathogen recognition receptor
QPCR	Quantitative polymerase chain reaction
QRDR	Quinolone resistance determining region
REA	Restriction endonuclease analysis
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
rRNA	Ribosomal RNA
RT	Reverse transcription
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
SHP	Stabilised hydrogen peroxide
sIgA	Secretory IgA
SLP	S-layer protein
TBE	Tris borate EDTA
TBS	Tris buffered saline
TCS	Two-component signal transduction system
TcdA	*C. difficile* toxin A
TcdB	*C. difficile* toxin B
TEA	Triethylamine
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
TLR	Toll-like receptor
Tm	Temperature of melting
TMA	Tetramethylammonium chloride
TMB	3,3’,5,5’-tetramethylbenzidine
TNF	Tumour necrosis factor
TSLP	Thymic stromal lymphopoeitin
UDP	Uridine diphosphate

1. Introduction

Clostridium difficile is a Gram-positive, obligately anaerobic, spore-forming bacillus. First isolated in 1935 from the stools of healthy neonates, it was named *Bacillus difficilis* due to the difficulty encountered while attempting to isolate this slow growing bacterium (Hall & O'Toole, 1935). *C. difficile* was first associated with infection in 1962, but there was little evidence to suggest that it was the primary pathogen in any of the infections (Smith & King, 1962). It was only in the late 1970s that the role of *C. difficile* in human infection was established.

Antibiotic-associated colitis (AAC) and its rare, severe form, pseudomembranous colitis (PMC), were identified in the 1950s and *Staphylococcus aureus* was the suspected pathogen (Pearce & Dineen, 1960). However, in 1974, Tedesco and colleagues identified PMC patients who had received prior clindamycin treatment (Tedesco *et al.*, 1974). Although no pathogen was isolated from any of the patients, this ruled out *S. aureus* as the cause because it was susceptible to clindamycin. In 1977, Bartlett and colleagues identified a clindamycin-resistant, toxin-producing *Clostridium* species as the cause of AAC in hamsters and even suggested *C. difficile* in particular (Bartlett *et al.*, 1977). The same year, a clostridial toxin that was neutralised by the *Clostridium sordellii* antitoxin was identified in the faeces of patients with PMC and even those with antibiotic-associated diarrhoea without colitis (Larson & Price, 1977; Rifkin *et al.*, 1977). In 1978, George and colleagues extended their search for the PMC pathogen by isolating all possible *Clostridium* species from the stools of affected patients (George *et al.*, 1978a; George *et al.*, 1978b). They observed that, of all the clostridia isolated, only pure cultures of *C. difficile* elicited the same cytotoxicity as that obtained with patient faeces and this was neutralised by the monovalent *C. sordellii* antitoxin. Thus, finally, the causative agent of PMC had been identified as *Clostridium difficile*. These results were confirmed by other investigators in humans and in animal models (Bartlett *et al.*, 1978b; Chang *et al.*, 1978a). Today, *C. difficile* is the most common cause of hospital-acquired and post-antibiotic diarrhoea (Rupnik *et al.*, 2009).

1.1. The bacterium and its identification

1.1.1. Morphology

Under the microscope, *C. difficile* cells appear as short heavy-bodied to long bacilli, 2 to 8 μm long and 0.5 μm wide, with elongated subterminal or nearly terminal spores that do not swell the bacilli (George *et al.*, 1979; Hafiz & Oakley, 1976; Hall & O'Toole, 1935; Smith & King, 1962). They are Gram-positive, but the stain tends to be lost in older cultures. They are also motile with few peritrichous flagella.

1.1.2. Media and colony characteristics

C. difficile produces white, flat, opaque, non-haemolytic colonies with irregular margins on reinforced clostridial agar and yellowish, lecithinase negative colonies with ground-glass appearance and filamentous edges on the preferred selective media used for its isolation like cycloserine cefoxitin fructose egg yolk agar (CCFA) and cylcoserine cefoxitin egg yolk agar (CCEY) (Brazier, 1993; George *et al.*, 1979; Hafiz & Oakley, 1976). The colonies produce a characteristic yellow-green or chartreuse fluorescence under UV light and have an unmistakable dung-like odour (George *et al.*, 1979; Wren, 2010). The addition of bile salts such as taurocholate or lysozyme to the medium facilitates the outgrowth of spores and is useful in environmental sampling (Wilcox *et al.*, 2000; Wilson *et al.*, 1982; Wren, 2010). Treatment of faecal samples with alcohol at a final concentration of 50% followed by the use of selective media enhances the recovery of *C. difficile* (Borriello & Honour, 1981; Fedorko & Williams, 1997). Further, rapid identification can be achieved by using a proline-aminopeptidase disc along with culture (Fedorko & Williams, 1997).

1.1.3. Antigen detection

Detection of toxin was the first diagnostic method employed for *C. difficile*; assays of toxicity in hamsters, vascular permeabilisation in rabbit skin and tissue culture neutralisation tests with *C. sordellii* antitoxin were used originally (Larson & Price, 1977; Rifkin *et al.*, 1977). Cytotoxicity neutralisation is the most accurate method to detect *C. difficile* toxin (Whittier *et al.*, 1993), but it is time-consuming and requires

facilities and expertise. Several commercial enzyme immunoassay (EIA) kits are available to detect *C. difficile* toxins directly from faecal samples. Kits like the VIDAS *C. difficile* toxin A assay (BioMerieux) and the *C. difficile* Tox-A-Test (Techlab) detect toxin A (Whittier *et al.*, 1993), but combined kits that detect both toxin A and toxin B such as the TOX A/B test (Techlab) (Aldeen *et al.*, 2000) and ImmunoCard Toxins A&B test (Meridian) (van den Berg *et al.*, 2005) are preferable due to the existence of A-B+ *C. difficile* strains (Borriello *et al.*, 1992). These assays are quick and easy to perform; the ImmunoCard assay detects toxin within 20 minutes. However, owing to variations in sensitivities and specificities and poor predictive values, it is preferable to use them in conjunction with culture.

The detection of the glutamate dehydrogenase (GDH) antigen is also possible using the *C.DIFF* CHECK-60 kit (Techlab); however, it detects both toxigenic and non-toxigenic *C. difficile* strains and needs to be followed by toxin detection (Snell *et al.*, 2004). A two-step GDH-toxin detection method is superior to EIA (Gilligan, 2008).

1.1.4. Gene detection

For a more accurate detection of *C. difficile*, PCR methods have been developed. Van der Berg and colleagues developed a real-time PCR method to amplify *tcdB* which correlated well with culture and cytotoxicity testing (van den Berg *et al.*, 2005). This is a suitable first-step identification method for *C. difficile*. The BD GeneOhm Cdiff also employs amplification of *tcdB* and correlates well with toxigenic culture (Barbut *et al.*, 2009a). A rapid real-time PCR method using fluorescence resonance energy transfer probes was developed by Sloan and colleagues, which was able to identify deletions in the *tcdC* gene of *C. difficile* isolates (Sloan *et al.*, 2008). This method is also highly sensitive and specific when compared to toxigenic culture. The most recent real-time PCR assay involves the detection of *tcdA*, *tcdB* and the *tcdC* deletion at position 117, which is characteristic of the hypervirulent ribotype 027 (de Boer *et al.*, 2010). These molecular methods are very accurate and can be used directly as screening tests prior to toxigenic culture.

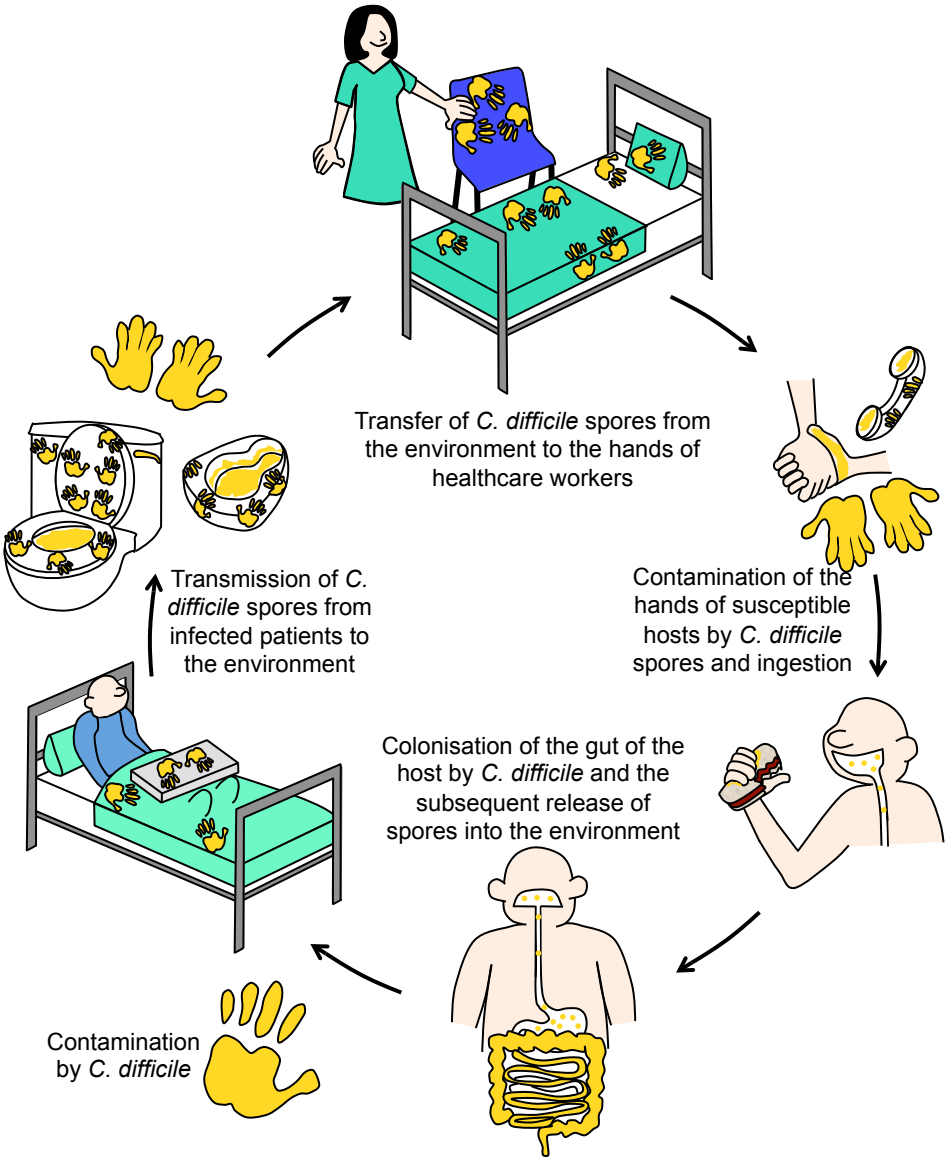
1.2. The disease

1.2.1. Acquisition of *C. difficile*

C. difficile is widely distributed in the environment. It has been isolated from soil, water and root vegetables (al-Saif & Brazier, 1996) and also from animals (Songer, 1996) and a variety of meat products (Weese *et al.*, 2009), suggesting its potential as a zoonotic and food-borne disease (Rupnik, 2007). Humans are also an important source of *C. difficile*. Asymptomatic carriage of the bacterium has been identified in adults and more commonly in neonates (Collignon *et al.*, 1993; Delmée, 2001). However, the main source of *C. difficile* are infected patients who release large numbers of vegetative cells and spores into their environment (Mulligan *et al.*, 1980; Wilcox *et al.*, 2003). This is especially significant in a nosocomial setting in which commonly touched surfaces and equipment and even the hands of healthcare workers can become contaminated and aid in the dissemination of *C. difficile* (Fekety *et al.*, 1981; Gerding *et al.*, 1995). Being transmitted by the faecal-oral route, the ingestion of *C. difficile* into the gut of the host is the primary step towards infection (Lyerly *et al.*, 1988; Worsley, 1998); contaminated hands and thus food are normally responsible for this. This transmission has been demonstrated in animals (Fekety *et al.*, 1980). Under suitable conditions in the gut, *C. difficile* is able to colonise the host, the spores germinate into vegetative cells that produce toxins and other virulence factors, and also spores, which are once again shed during diarrhoea (Poxton *et al.*, 2001). In this way, the cycle of transmission of *C. difficile* from host to environment and vice-versa continues (Fig. 1.1). The reservoirs of *C. difficile* and its environmental spread are discussed in greater detail in Chapter 4 (4.1.1-4.1.2).

Clostridium difficile infection (CDI), previously known as *Clostridium difficile*-associated disease (CDAD) is primarily a nosocomial occurrence, sometimes referred to as healthcare-associated CDI (HA-CDI), but community-associated CDI (CA-CDI) is not unheard of (Limbago *et al.*, 2009). Although it can be difficult to distinguish between the two, the incidence of community-associated cases has been increasing in recent years (Norén *et al.*, 2004; Riley *et al.*, 1995).

Fig. 1.1. Transmission of *C. difficile* in a nosocomial setting



C. difficile can be acquired from the environment, primarily in the form of spores, by direct ingestion with food or from contaminated equipment. Once established in the gut of the host, the bacterium multiplies and produces spores, which are shed into the environment. In infected patients, frequent diarrhoea can result in the release of large numbers of spores. If the environment is not effectively decontaminated, the spores can persist for long periods of time and transmission can occur through the contamination of objects and also the hands of health care workers. From these sources, *C. difficile* can be transmitted to other susceptible individuals, who could in turn become a source of infection. In this way, the faecal-oral transmission of *C. difficile* in the environment and the host continues. Adapted from http://www.infectionprotection.org.uk/stages/c-diff.html.

1.2.2. Risk factors

1.2.2.1. Exposure to antibiotics

'Colonisation resistance' by the normal indigenous gut microbiota is key in preventing colonisation by *C. difficile* (Wilson, 1993). Thus, its disruption by antibiotics and the subsequent lack of competition is the most important risk factor for CDI (Bartlett *et al.*, 1980; Kelly & LaMont, 1998). The role of exposure to antibiotics in *C. difficile* infection was first demonstrated in the hamster model; even pre-exposure to vancomycin, which was considered to be protective (Fekety *et al.*, 1979), followed by the oral administration of *C. difficile* resulted in disease (Larson *et al.*, 1978). A range of antibiotics has been shown to impact the susceptibility of the host to *C. difficile* (Bartlett *et al.*, 1978a; Bartlett *et al.*, 1980). When first identified, CDI was synonymous with the use of clindamycin and its effect was demonstrated in animal models (Chang *et al.*, 1978a; Tedesco *et al.*, 1974). However, the replacement of clindamycin by third-generation cephalosporins and broad-spectrum penicillins led to them being implicated most frequently in CDI (Bartlett, 2008; Brown *et al.*, 1990). Fluoroquinolones were initially considered to be unlikely suspects in CDI due to their minimal disruptive effects on the anaerobic microbiota of the gut (Golledge *et al.*, 1992; Riley *et al.*, 1991). However, the emergence of resistant epidemic strains in the last decade has caused a shift in perspective (Kuijper *et al.*, 2006; McDonald *et al.*, 2005). The duration of antibiotic exposure has also been identified as a risk factor for CDI; increased duration and use of multiple antibiotics like quinolones, cephalosporins, clindamycin and macrolides can increase incidence (Brown *et al.*, 1990; Pépin *et al.*, 2005a). The role of fluoroquinolones in recent epidemics caused by a hypervirulent *C. difficile* strain is discussed in greater detail in Chapter 6 (6.1.3).

1.2.2.2. Age

C. difficile colonisation of neonates and the lack of disease in this group is well-known (Behrman & Donta, 1982; Collignon *et al.*, 1993; Stark *et al.*, 1982) and asymptomatic carriage by healthy adults is common (Delmée, 2001; Kato *et al.*, 2001; Ozaki *et al.*, 2004). Historically, *C. difficile* infection, was a disease of the

elderly. A significant risk factor was age above 65 years (McFarland & Stamm, 1986; Pépin *et al.*, 2004; Sunenshine & McDonald, 2006), possibly due to a combination of confounding factors such as the presence of underlying conditions, increased antibiotic treatment, the inability to mount an effective immune response and alterations in the normal gut microbiota (Hopkins & Macfarlane, 2002; McFarland & Stamm, 1986).

More recently, however, disease in previously low-risk groups has been identified. These include children, peripartum women and young healthy adults with no prior exposure to antibiotics or recent hospitalisation (Benson *et al.*, 2007; Centers for Disease Control and Prevention, 2005). Most of these infections are likely to be community-acquired and linked with less morbidity, possibly due to the age and health status of the individuals (Karlström *et al.*, 1998; Naggie *et al.*, 2010), but severe disease does occur (Centers for Disease Control and Prevention, 2005).

1.2.2.3. Underlying disease

The presence of underlying conditions and immunosuppression increase with age and so does the risk of CDI (Pépin *et al.*, 2004). Underlying conditions can include gastrointestinal, pulmonary and urinary tract infections, renal disease, cellulitis, cancer, anaemia, diabetes mellitus and even the need for emergency surgery (Changela *et al.*, 2004; Harbarth *et al.*, 2001; Kyne *et al.*, 2002). Notably, similar conditions add to the risk of infection in children: underlying gastrointestinal pathology and surgery, renal disease, cancer and hypogammaglobulinaemia (Benson *et al.*, 2007; McFarland *et al.*, 2000). High prevalence of *C. difficile* infection has also been identified in patients with irritable bowel disease (IBD) and increased mortality is associated with patients with IBD and CDI (Ananthakrishnan *et al.*, 2008; Pituch, 2009). CDI has also been associated with HIV infection, norovirus outbreaks and seasonal influenza (Polgreen *et al.*, 2010; Sanchez *et al.*, 2005) (Svraka *et al.*, 2010), although this link is most likely due to the compromised immune status of the host and antimicrobial use. The severity of underlying conditions was thought to play an indirect role in CDI by aggravating other risk

factors such as the duration of hospitalisation and bacterial exposure (McFarland *et al.*, 1990) but it has now been observed to play a more direct role in disease, possibly by impairing the immune response of the host (Kyne *et al.*, 2002).

1.2.2.4. Duration of hospitalisation

Hospitalisation is an important risk factor in CDI as hospitals themselves are a source of infection (Kaatz *et al.*, 1988). The rate of colonisation among hospitalised adults was observed to be more than 20% and the risk of acquiring *C. difficile* was directly proportional to the length of stay (Johnson & Gerding, 1998; McFarland *et al.*, 1989). Patients moved between wards were also found to be at increased risk, possibly due to increased duration of hospitalisation and increased exposure to antibiotics and environmental contamination (Starr *et al.*, 2003). However, not all studies agree with these observations; reports in which the duration of hospitalisation has no implication on CDI have also been published (Brown *et al.*, 1990).

1.2.2.5. Gastric acid suppression

Proton pump inhibitors (PPIs) that lower gastric acidity are often suggested as a risk factor (Cunningham & Dial, 2008; Dial *et al.*, 2006). Their effect in infection and subsequent inflammation was demonstrated in the animal model and the risk associated with them was comparable to that associated with antibiotics (Kaur *et al.*, 2007). Vegetative cells of *C. difficile* were able to survive in the gastric contents of patients receiving PPIs at a pH above 5 and this could perhaps aid survival of the bacteria and increase the risk of infection (Jump *et al.*, 2007). However, PPIs did not affect spore germination and the mechanism by which they could promote CDI is still unknown (Nerandzic *et al.*, 2009). In most investigations so far the association of PPIs with CDI has either not been demonstrated (Loo *et al.*, 2005; Wilcox *et al.*, 2008) or has been weak at best (Muto *et al.*, 2005).

1.2.2.6. Other interventions

Treatments such as anti-neoplastic drugs, diuretics, stool softeners, antacids, laxatives and immunosuppressive agents like corticosteroids have also been

associated with *C. difficile* infection and carriage (Anand & Glatt, 1993; Faris *et al.*, 2010; McFarland *et al.*, 1990; Raveh *et al.*, 2006). In-hospital procedures like the administration of enemas, nasogastric tubes, endoscopy and surgery have also been linked with CDI, but these are most likely to reflect environmental contamination (McFarland *et al.*, 1990; Spencer, 1998). In one report, underlying conditions and treatments individually were not found to affect CDI, but a combination of two or more of these significantly increased the frequency of CDI (Wiström *et al.*, 2001)

1.2.2.7. Strain type

C. difficile strains can exhibit a range of pathogenic potential; a correlation between the type of strain colonising a patient, its ability to produce toxins and the symptoms of disease has been observed (Wren *et al.*, 1987), but a lack of association has also been documented (Wilson *et al.*, 2010). Certain hypervirulent strains have recently been identified like ribotype 027 (Pépin *et al.*, 2005b), ribotype 078 (Jhung *et al.*, 2008) and ribotype 017 (Arvand *et al.*, 2009). Others like ribotypes 001 and 106 have also been associated with severe disease (Arvand *et al.*, 2009; Sundram *et al.*, 2009). However, the changing epidemiology suggests that other strains have similar abilities to cause disease (Bauer *et al.*, 2010; Rupnik *et al.*, 2003b; Taori *et al.*, 2009). Moreover, the pathogenicity of the infecting strain alone does not determine the severity of disease; host factors are also deemed important (McFarland *et al.*, 1991).

1.2.2.8. Host immune response

The ability of the host to mount an immune response against *C. difficile* is believed to play a significant role in the severity and recurrence of disease. Antibody production against *C. difficile* is possibly stimulated early in life (Kelly & Kyne, 2011); neonates are exposed to the bacterium in the environment (Bolton *et al.*, 1984) or even in the birth canal (Hafiz *et al.*, 1975). It has also been suggested that antibodies to *C. difficile* can be acquired from colostrum (Wada *et al.*, 1980). This response is possibly enhanced by environmental exposure later on in life. Thus, not surprisingly, most adults have serum IgG and IgA against the toxins and other antigens of *C. difficile* (Pantosti *et al.*, 1989; Sánchez-Hurtado *et al.*, 2008).

The correlation between antibody levels and the clinical course of infection has been demonstrated in several studies; short acute infection, asymptomatic carriage and a decreased risk of relapse were observed in patients with higher levels of serum IgG, IgM and IgA against the toxins and cell-somatic antigens and more faecal IgA (Aronsson *et al.*, 1985; Mulligan *et al.*, 1993; Warny *et al.*, 1994). In one study, serum IgA was found to be responsible for the neutralisation of toxin A in the convalescent phase of infection (Johnson *et al.*, 1995). In a course-related study, although the levels of IgA, IgM and IgG between patients were found to be similar at the time of admission to hospital, by the time of colonisation with *C. difficile*, the IgG response to the toxins and other antigens were much higher in asymptomatic carriers and dropped significantly in those who developed infection (Kyne *et al.*, 2000). Also, among patients who showed no differences in antibody levels at the onset of diarrhoea, increased levels of IgM on day 3 and of serum IgA and IgG on day 12 were observed in those who did not relapse (Kyne *et al.*, 2001). The strength and time of the immune response to *C. difficile* appear to be critical; a greater anamnestic response can avert symptoms and an early response in infection can prevent recurrence (Kelly & Kyne, 2011).

Antibodies to non-toxin antigens of *C. difficile* have also been thought to play a role in infection. In an early study, higher levels of IgG to surface-layer proteins (SLPs) were seen in the convalescent phase of infection and the highest levels were seen in patients who later relapsed (Pantosti *et al.*, 1989). In another study, it was found that asymptomatic carriers had higher levels of IgM to SLPs and patients who could not mount an IgM response suffered recurrent infection (Drudy *et al.*, 2004). Antibodies against flagella proteins FliC and FliD and surface-associated proteins Cwp66 and Fbp68 were found to be lower in symptomatic patients as compared to controls when there was no difference in antibodies to the toxins, suggesting that they might have a role in colonisation (Péchiné *et al.*, 2005a).

A study of the immune cell populations in the colonic biopsies of patients with diarrhoea not caused by *C. difficile* and those with mild or severe CDI revealed that

there was a significant reduction in the B/plasma cells and macrophages in the latter, while the numbers of T cells did not vary between the groups (Johal *et al.*, 2004b). A specific reduction in IgA producing cells and an increase in IgG producing cells was observed in patients with CDI and especially in those with pseudomembranous colitis. Further, both in this study and others, biopsies of patients who relapsed contained fewer IgA producing cells than those of patients who did not (Wroe, 2009). Thus, recurrence of CDI may be associated with a reduction in IgA producing cells.

1.2.3. Clinical presentations

CDI is characterised by the presence of diarrhoea which is normally observed during or shortly after antibiotic therapy, but diarrhoea can occur post cessation of antibiotic therapy (Kelly & LaMont, 1998). The clinical presentation of disease, however, can range from asymptomatic carriage to mild disease to fulminant colitis. In mild cases of CDI, diarrhoea is watery, does not contain blood or mucus and normally ceases with stopping antibiotic therapy, and for asymptomatic carriers, treatment is not recommended. When CDI is more serious, colitis coupled with abdominal pain, malaise, fever, dehydration and delirium can occur (Kelly & LaMont, 1998; Starr, 2005). In this form of the disease, pseudomembranes are usually absent. When pseudomembranous colitis occurs, the symptoms are the same, but are much more severe. Classic pseudomembranes contain raised yellow necrotic plaques scattered over the colonic mucosa (Castagliuolo & LaMont, 1999; Hafiz & Oakley, 1976; Kelly & LaMont, 1998). Fulminant colitis is rare (Dallal *et al.*, 2002) but exhibits the most intense complications such as perforation and toxic megacolon. Thus, not surprisingly, mortality rates of about 40% are associated with fulminant colitis (Sailhamer *et al.*, 2009). High fever, chills, leukocytosis and hypoalbuminemia are also commonly observed (Bartlett, 2008; Bartlett, 2010a; Kelly & LaMont, 1998).

1.2.4. Diagnosis

The laboratory diagnosis of CDI in a patient presenting with diarrhoea and other symptoms can be confirmed by the detection of toxins. The cytotoxicity assay involving the exposure of fibroblasts to *C. difficile* toxin followed by their

neutralisation with *C. sordellii* antitoxin (Chang *et al.*, 1978b) was the 'gold standard' for testing until the introduction of an enzyme linked immunoassays for toxin A or both toxin A and toxin B (Bartlett, 2008; Bartlett, 2010b; Lyerly *et al.*, 1983). Today, a variety of commercial immunoassays and gene detection kits exist for the rapid detection of *C. difficile* (1.1.3-1.1.4), but toxigenic culture remains the most accurate method for the laboratory diagnosis of *C. difficile* (Delmée *et al.*, 2005; Reller *et al.*, 2007).

The clinical diagnosis of CDI does not involve sigmoidoscopy or colonoscopy, unless the diagnosis is doubtful (Kelly & LaMont, 1998). In fact, it has been shown that nurses are able to diagnose CDI accurately from information like recent use of antibiotics, presence of fever and characteristic 'clostridial' odour (Johansen *et al.*, 2002; Wilcox, 2007). For pseudomembranous colitis, direct visualisation by sigmoidoscopy or colonoscopy is required, but due to the lack of specificity, they must be used in conjunction with laboratory techniques to confirm the diagnosis (Gerding *et al.*, 1995). Flexible sigmoidoscopy is recommended for hospitalised patients presenting symptoms of CDI in whom toxins cannot be detected by laboratory tests (Johal *et al.*, 2004a).

1.2.5. Treatment and outcomes

1.2.5.1. Conventional antimicrobial therapy

The treatment of *C. difficile* infection involves the discontinuation of the inciting antibiotic, followed by the administration of antimicrobials, despite their drawbacks.

In 20% of CDI patients symptoms are resolved without treatment, but for those requiring antibiotic therapy, metronidazole and vancomycin are the drugs of choice (Starr, 2005). Successful treatment with both metronidazole and vancomycin has been observed (Burdon *et al.*, 1979; Keighley *et al.*, 1978; Pashby *et al.*, 1979). Metronidazole was selected as the first-line treatment for CDI due to the higher cost of vancomycin and its potential to select for vancomycin-resistant enterococci (Gerding, 2009). However, the acquisition of *C. difficile* during metronidazole

treatment was also observed and it was suggested that vancomycin be the first-line treatment (Mogg *et al.*, 1979). The debate between these two antibiotics was resolved by a comparative study, which showed that both were suitable for the treatment of mild CDI, but vancomycin is superior for the treatment of severe disease (Zar *et al.*, 2007). In patients with fulminant colitis, vancomycin treatment can increase the odds of survival by 4-fold (Sailhamer *et al.*, 2009), whereas metronidazole has been related to increased failure of therapy and recurrence (Musher *et al.*, 2005; Pepin *et al.*, 2005; Wilson *et al.*, 2010). However, this advantage of vancomycin in severe disease was not demonstrated in CDI caused by the hypervirulent ribotype 027 (Pepin, 2008). Oral vancomycin is preferred for the treatment of pregnant and breast-feeding women and those who fail to respond to metronidazole treatment (Razavi *et al.*, 2007). Both antibiotics are equally effective in the treatment of first recurrences, irrespective of the agent used for the resolution of the initial episode (Pépin *et al.*, 2006). A combination of metronidazole and vancomycin may be used in critically ill patients (Kelly & LaMont, 1998).

1.2.5.2. New alternatives

Antibiotics other than vancomycin and metronidazole have been tested against *C. difficile*. These include bacitracin, teicoplanin, fusidic acid, nitaxozanide, rifaximin, linezolid and ramoplanin (Baines *et al.*, 2011; Monaghan *et al.*, 2008). Teicoplanin was found to be slightly better than vancomycin and metronidazole (Nelson, 2007), while nitazoxanide, which is normally used to treat parasite-infections (Monaghan *et al.*, 2008), bacitracin and fusidic acid were not found to be of added advantage (Gerding & Johnson, 2010). Rifaximin and rifampin have been found to be useful in preventing recurrence of disease when administered immediately after vancomycin therapy, but resistance to this antibiotic has also been observed (Johnson *et al.*, 2007; Lagrotteria *et al.*, 2006; O'Connor *et al.*, 2008). REP3123, a narrow-spectrum antibacterial agent, demonstrated high selectivity for *C. difficile* and was more efficient at inhibiting the production of toxins and spores in *C. difficile* strains when compared to vancomycin and metronidazole (Citron *et al.*, 2009; Ochsner *et al.*, 2009). The most recent antibiotic tested against *C. difficile* is fidaxomicin (OPT-80),

which appears to have a higher clinical cure than vancomycin (Poxton, 2010). Fewer relapses have also been noted with fidaxomicin, probably due to its minimal effects on the normal gut microbiota (Tannock *et al.*, 2010).

1.2.5.3. Surgery

Prompt surgical intervention for patients with fulminant colitis who do not respond to aggressive antimicrobial therapy can increase the chances of survival (Kelly & LaMont, 1998; Sailhamer *et al.*, 2009). The main aim of surgery is to prevent complications like bowel perforation and faecal peritonitis and thus, avoid the high morbidity and mortality associated with these conditions (Klingler *et al.*, 2000). Despite this, the mortality associated with surgery is high due to the ill health of patients requiring it (Kelly & LaMont, 1998).

1.2.5.4. Toxin neutralisation

The alternate therapies for CDI are aimed at removal of the toxins from the gut to prevent tissue damage, restoration of the normal microbiota and improvement of immunity.

C. difficile toxin A and toxin B can bind to anion-exchange resins cholestyramine and cholesterol, but these toxin-binding compounds were found to be clinically ineffective (Thompson, 2008) especially since cholestyramine binds to vancomycin (McFarland *et al.*, 2000). Tolevamer, an anionic polymer that binds both toxins, was found to be as effective as vancomycin in the treatment of mild to moderate CDI in initial trials (Louie *et al.*, 2006). However, it was later found to be inferior to conventional antimicrobial therapy (Gerding & Johnson, 2010) possibly because it was unable to neutralise the highly cytotoxic toxin B (Baines *et al.*, 2009).

The neutralisation of toxicity using bovine immunoglobulin has been demonstrated in hamsters (Lyerly *et al.*, 1991). This effect was also observed in humans (Warny *et al.*, 1999) but no large-scale trials have been conducted so far. The whey protein in immunised cow's milk has also shown potential for use in treatment, but the results remain inconclusive so far (Gerding & Johnson, 2010; Young *et al.*, 2007).

1.2.5.5. Probiotic therapy

Since the perturbation of normal intestinal microbiota leads to *C. difficile* infection, its restoration is important, especially following antibiotic therapy. Investigations into the use of probiotics containing yeasts like *Saccharomyces boulardii*, or bacteria such as *Lactobacillus* species in *C. difficile* treatment have been inconclusive; although there may be some benefit in secondary prevention, there is also a risk of fungaemia and bacteraemia, especially in immunocompromised and chronically ill individuals (Miller, 2009; Tung *et al.*, 2009; Verna & Lucak, 2010). The less appealing form of probiotic therapy involves faecal transplants. This method has been mainly used for patients with recurrent CDI and has been found to be highly effective and safe (MacConnachie *et al.*, 2009; van Nood *et al.*, 2009). Another approach for biotherapy is to induce colonisation by a non-toxigenic *C. difficile* strain. Administration of a non-toxigenic strain after antimicrobial treatment has been successful in hamsters (Borriello & Barclay, 1985; Merrigan *et al.*, 2009), but treatment in humans could be complicated by ongoing antibiotic treatment and the susceptibility of the non-toxigenic strains to it. In phase I clinical trials using a non-toxigenic strain, the organism was found to be well-tolerated after vancomycin therapy and was isolated from faecal samples of all the subjects, confirming colonisation (Tatarowicz *et al.*, 2010). Further trials will determine the use of this approach in CDI treatment.

1.2.5.6. Antibody therapy and vaccines

Active and passive immunisation against *C. difficile* toxins would be most suitable to protect against CDI. Preliminary studies with a parenteral vaccine containing toxoid A and toxoid B have been shown to resolve recurrent diarrhoea with a simultaneous increase in serum IgG levels against both the toxins (Sougioultzis *et al.*, 2005). Transcutaneous injection of toxoid A in conjunction with cholera toxin in mice induced systemic and mucosal responses, suggesting an alternate strategy for immunisation (Ghose *et al.*, 2007). More recently, oral immunisation of hamsters with spores of *Bacillus subtilis* expressing the cell-binding domains of toxin A and toxin B was found to confer protection from infection (Permpoonpattana *et al.*,

2011). Also, an immune response to toxin A alone was found to be effective against CDI. Passive immunity with individual monoclonal antibodies against toxin A and toxin B when added to antibiotic treatment was able to significantly reduce the risk of recurrence (Lowy *et al.*, 2010). Administration of monoclonal antibody to toxin A alone did not reduce the rate of recurrence (Leav *et al.*, 2010). Whether monoclonal antibodies will support therapy or be prophylactic is yet to be determined (Kyne, 2010). Results from one study suggested that the oral administration of a monoclonal antibody to toxin A conjugated to an inert support would be more effective at the neutralisation of toxin A than free antibody (Sutton *et al.*, 2008). The only current in-use antibody therapy for CDI is intravenous immunoglobulin, which appears to be beneficial in severe disease and recurrences, but current data is insufficient to support its widespread use (O'Horo & Safdar, 2009).

1.2.5.7. Carriage and recurrence

The outcome of colonisation by *C. difficile* is dependent on a combination of the above-mentioned risk factors, that is, it can result in symptomatic disease or asymptomatic carriage. Patients who have acquired *C. difficile* during hospitalisation can become asymptomatic carriers (McFarland *et al.*, 1989). Alternately, following symptomatic disease and treatment, recurrence of infection can be observed. Recurrence can occur in 20 to 30% of patients and could either be a relapse caused by the same strain or a re-infection with a new strain (Starr, 2005; Wilcox & Spencer, 1992). The treatment of recurrences involves either supportive management without antibiotics or treatment with the antibiotic used for the initial episode (Maroo & Lamont, 2006). Recurrence itself is a risk factor; a single recurrence increases the risk for subsequent recurrences (Maroo & Lamont, 2006).

1.2.6. Prevention and control

Two main aspects of the prevention and control of *C. difficile* infection are the elimination of the source of infection by effective decontamination of the environment and the restriction on the use of antibiotics that are not only an important risk factor for disease, but also aid the selection of resistance in

microorganisms (Blondeau, 2009; O'Connor *et al.*, 2009). The role of appropriate and thorough environmental disinfection in restricting the spread of *C. difficile* and even curbing outbreaks is discussed in detail in Chapter 4 (4.1.6-4.1.8) including the agents and strategies most suitable for this purpose. Good antibiotic stewardship can also decrease the incidence of symptomatic disease and contribute to control of CDI (Brown *et al.*, 1990; Dellit *et al.*, 2007; Vonberg *et al.*, 2008). The complete restriction of high-risk antibiotics like fluoroquinolones has been shown to be effective in controlling an outbreak by an epidemic strain (Kallen *et al.*, 2009). However, not just targeting high-risk antibiotics, but optimisation of the use of antibiotics is essential to the prevention and control of CDI (Valiquette *et al.*, 2007).

1.3. Typing schemes

1.3.1. Early methods

The early typing schemes for *C. difficile* were based on the phenotype of the organisms. Crossed immunoelectrophoresis, analysis of protein patterns by SDS-PAGE and plasmid profiles proved to be useful in identifying dominant strains in outbreaks and differentiating them from sporadic isolates (McKay *et al.*, 1989; Poxton *et al.*, 1984; Tabaqchali *et al.*, 1984; Wüst *et al.*, 1982). Bacteriophage and bacteriocin typing was also able to differentiate *C. difficile* strains from one another and from other clostridia (Sell *et al.*, 1983). In 1985, Delmée and colleagues developed serogrouping (Delmée *et al.*, 1985). They initially identified six serogroups, two of which consisted of non-toxigenic strains and two of which were found to be outbreak-specific. A comparison of serogrouping with SDS-PAGE protein profiles showed that both methods could be used for typing *C. difficile* strains (Delmée *et al.*, 1986). Further, dominant serogroups and sub-types in serogroups with varying protein profiles were identified; serogroup A consisted of isolates having 12 different profiles. Serogrouping is often used as a standard for comparison with other schemes (Brazier, 2001). Surface-layer typing was another phenotypic method used to type *C. difficile*, which exploited inter-strain variability in the sizes of

the low and high molecular weight SLPs (McCoubrey & Poxton, 2001; McCoubrey *et al.*, 2003).

1.3.2. REA

The use of restriction endonuclease analysis (REA) in *C. difficile* typing was first demonstrated by Kuijper and colleagues (Kuijper *et al.*, 1987). They used *Hin*dIII to restrict whole cell-DNA and observed distinguishing restriction patterns for different strains on agarose gels. The method correlated well with the protein profiles of the strains. Analysis of a large number of isolates by this method identified a total of 206 REA types that grouped into 75 groups (Clabots *et al.*, 1993). This is a rapid and highly discriminatory method for typing *C. difficile* but inter-laboratory comparisons are difficult and is not amenable to image analysis. REA analysis using *Cfo*I has also been used for typing *C. difficile* strains (Devlin *et al.*, 1987).

1.3.3. Ribotyping

Ribotyping is a method based on the variability in length of the 16S-23S intergenic spacer regions in different alleles and was first suggested as a typing method for *C. difficile* by Gürtler (Gürtler, 1993). By amplifying these regions of DNA and running them on long denaturing polyacrylamide gels, he identified different banding patterns for different strains and was able to group 24 strains into 14 ribotypes. Ribotyping was found to be a simple reproducible method to study a large number of isolates (Cartwright *et al.*, 1995). In 1996, O'Neill and colleagues modified Gürtler's method making it faster and easier (O'Neill *et al.*, 1996). They extracted DNA by boiling with a resin, re-designed the primers to give bands between 260 and 585 bp and used high resolution agarose for greater discrimination. By 1996, 116 ribotypes were defined (Stubbs *et al.*, 1999). A modification of the primers used for ribotyping by Bidet and colleagues was observed to further improve the readability and stability of the bands obtained (Bidet *et al.*, 1999; Bidet *et al.*, 2000). Ribotyping is the method of choice for *C. difficile* typing in the UK and Europe (Brazier, 2001). More recently, capillary gel electrophoresis-based PCR ribotyping has been developed, which overcomes the problem of inter-laboratory variations (Indra *et al.*, 2008).

1.3.4. Toxinotyping

Toxinotyping is a method based on the inter-strain variability in the toxin genes, *tcdA* and *tcdB* (Rupnik *et al.*, 1997). These variations are concentrated in the 3' third of *tcdA* (A3 fragment) and the 5' third of *tcdB* (B1 fragment). The restriction of these DNA regions with enzymes like *Eco*RI, *Acc*I and *Hin*cII formed the basis for toxinotyping (Rupnik *et al.*, 1998). The method correlated well with PFGE, serogrouping and ribotyping, although several ribotypes may group within a single toxinotype (Rupnik *et al.*, 2001). Twenty-five toxinotypes have been identified so far.

1.3.5. PFGE

Pulsed-field gel electrophoresis (PFGE) involves the analysis of the whole genome of an organism after restriction using infrequent cutting enzymes like *Sma*I and *Sac*II (Brazier, 2001). PFGE typing of *C. difficile* correlates well with serogrouping (Kato *et al.*, 1994) and can be even more discriminatory than ribotyping (Bidet *et al.*, 1999). It is often considered to be the gold standard for typing pathogenic bacteria. Using a modification to the method seven sub-types in PCR ribotype 001 isolates were identified (Gal *et al.*, 2005). PFGE is commonly used in North America and the strains are assigned North American pulsed-field (NAP) types (Tenover *et al.*, 2011).

1.3.6. AP-PCR

Arbitrarily-primed polymerase chain reaction (AP-PCR) involves the amplification of non-specific fragments of DNA using short primers under low-stringency conditions (Killgore & Kato, 1994; Wilks & Tabaqchali, 1994; Wullt *et al.*, 2003a). It is more discriminatory than immunoblotting, but not as reproducible as ribotyping.

1.3.7. MLST and MLVA

Multilocus sequence typing (MLST) was used to determine epidemiological links between *C. difficile* isolates (Lemée *et al.*, 2004). By sequencing the toxin genes and their positive regulator, the binary toxin genes and other genes involved in virulence, several *C. difficile* clones were identified (Lemée *et al.*, 2005). Multilocus variable-number tandem-repeat analysis (MLVA) has also been shown to be useful for *C.*

difficile typing (Marsh *et al.*, 2006). By comparing seven variable regions on the *C. difficile* genome, groups of strains were found, which corresponded well to REA groups. MLST and MLVA used in conjunction were very useful to identify genetic lineages and the genetic relationships between them (Marsh *et al.*, 2010).

1.3.8. *slpA*ST

The inter-strain differences in the variable region of the *slpA* gene of *C. difficile* were found to be sufficiently discriminatory to be used as a typing scheme (Karjalainen *et al.*, 2002). Surface-layer protein A gene sequence typing (*slpA*ST) involves PCR amplification of the variable region of *slpA*, followed by sequencing or RFLP. It correlated well with serogrouping: the *slpA* variable region was 100% identical in strains within a serogroup, while inter-serogroup identity was low. It is a useful genotyping tool (Joost *et al.*, 2009; Kato *et al.*, 2010; Ní Eidhin *et al.*, 2006).

1.4. Virulence factors

1.4.1. Toxin A and toxin B

Toxin A (TcdA) and toxin B (TcdB) of *C. difficile* are its most important virulence factors. They belong to the family of 'large clostridial cytotoxins' (Schirmer & Aktories, 2004) which also includes the haemorrhagic and lethal toxins of *C. sordellii* and the α-toxin of *C. novyi*. The *C. difficile* exotoxins TcdA and TcdB have molecular weights of 308 kDa and 269 kDa, respectively (Barroso *et al.*, 1990; Dove *et al.*, 1990; Sauerborn & von Eichel-Streiber, 1990). Traditionally, TcdA is termed an enterotoxin, while TcdB is termed a cytotoxin (Taylor *et al.*, 1981). TcdA does, however, possess cytotoxic activity, but it is a 100 to 1000-fold less than that of TcdB; TcdB was believed to have no enterotoxic activity (Donta *et al.*, 1982; Sullivan *et al.*, 1982), but more recently it has been observed that TcdB can be as enterotoxic as TcdA (Savidge *et al.*, 2003). Both toxins exhibit high structural and functional similarity (Just *et al.*, 1995b; Lyerly *et al.*, 1986; Taylor *et al.*, 1981). They are encoded and regulated by genes of the pathogenicity locus (PaLoc) of *C. difficile*, which are described in Chapter 3 (3.13).

1.4.1.1. Structure

Investigations into the structures of *C. difficile* TcdA and TcdB revealed functional dualism with a C-terminal binding domain and an N-terminal catalytic domain (von Eichel-Streiber *et al.*, 1992). A middle hydrophobic region was then identified (Barroso *et al.*, 1994). This tripartite toxin structure has now been replaced with a four-domain ABCD structural model: the glucosyltransferase domain, the cysteine protease domain, the translocation domain and the receptor-binding domain (Fig. 1.2) (Albesa-Jové *et al.*, 2010).

The N-terminal of the toxins is responsible for their catalytic activity. In this region the amino acid residues from 516 to 546 are structurally and functionally important for toxicity (Hofmann *et al.*, 1997; Soehn *et al.*, 1998). The four conserved cysteine residues in the hydrophobic region play a role in endocytosis (Barroso *et al.*, 1994). The C-terminal of the toxins contains short combined repetitive oligopeptides (CROPs) (von Eichel-Streiber *et al.*, 1992) and mediates cell-binding (Sauerborn *et al.*, 1997). The translocation domain has a minimal pore forming region from residues 830 to 990 and the residues from 1501 until the CROPs are essential for translocation (Genisyuerek *et al.*, 2011). TcdA and TcdB both lack signal peptides for extracellular secretion (von Eichel-Streiber & Sauerborn, 1990).

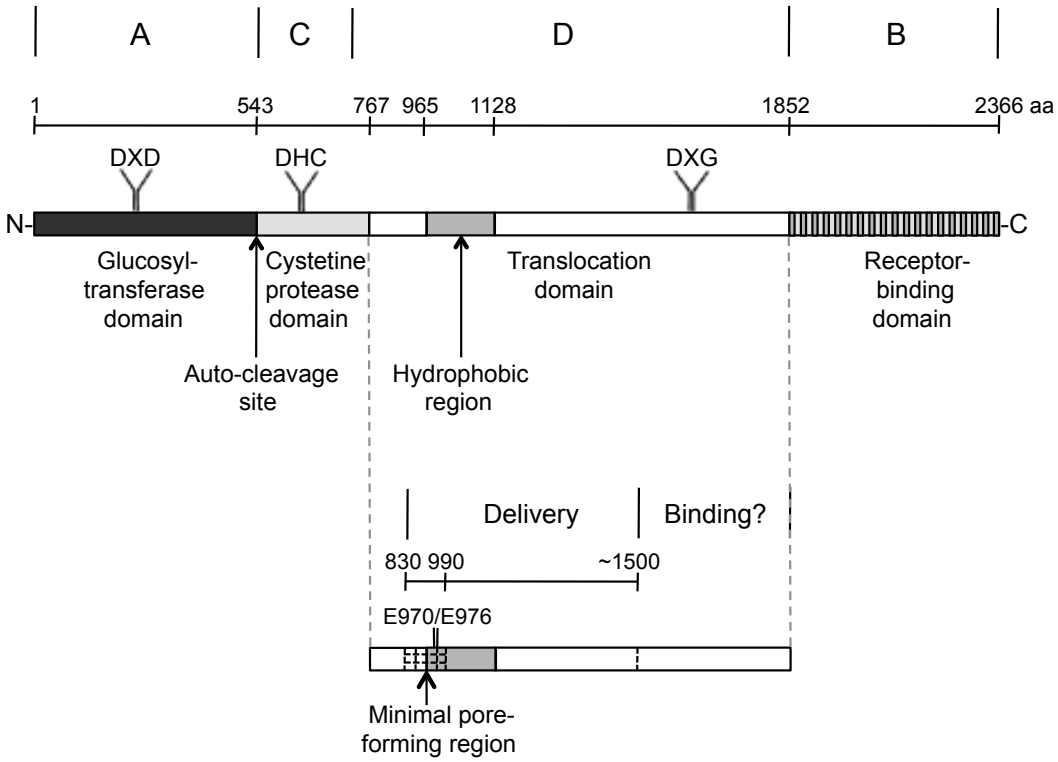
1.4.1.2. Cell-binding

TcdA and TcdB must be internalised via receptor-mediated endocytosis (Barth *et al.*, 2001) and then processed to reach the cytosol where they can elicit cytotoxic effects (Voth & Ballard, 2005).

Toxin-receptor interaction is the first step in the process of entry of toxins into cells. Carbohydrate receptors for TcdA, with a Galβ1-4GlcNAc core, were identified in the brush border membranes of hamsters (Krivan *et al.*, 1986) and on human intestinal epithelial cells (Tucker & Wilkins, 1991). A receptor for TcdB has not yet been identified (Voth & Ballard, 2005). It has been shown that TcdB exhibits biological activity on intestinal epithelial cells only when applied basolaterally, suggesting that

unlike TcdA receptors, its receptors are present only on the basolateral surface of epithelial cells (Sutton *et al.*, 2008). This supports the hypothesis that epithelial damage by toxin A or physical damage to the epithelium is required prior to TcdB-mediated cytotoxicity (Lyerly *et al.*, 1985).

Fig. 1.2. ABCD domain structure of clostridial glucosylating toxins



The ABCD model of clostridial glucosylating toxins is shown with *C. difficile* TcdB as an example. The glucosyltransferase domain (A, residues 1-543) is located at the N-terminus. The binding domain (B) at the C-terminus is comprised of repetitive sequences (CROPs). The cysteine protease domain (C, residues 544-767), characterised by the DHC domain, is involved in the processing and cleavage of the toxin. The DXG domain in the translocation domain (D) is part of the aspartate protease domain originally suggested to be involved in cleavage of the toxin. The hydrophobic region in the translocation domain (residues 956-1128) is involved in pore formation. This overlaps with the newly identified minimal-pore forming region (residues 830-990) in which the glutamate residues at positions 970 and 976 are crucial for pH-dependent toxin uptake. The residues 830-1500 are essential for translocation of the catalytic domain into the cytosol, while the residues 1501-1851 have been suggested to form an additional domain, possibly involved in binding. Adapted from Schirmer & Aktories, 2004 and Jank & Aktories, 2008.

1.4.1.3. Membrane translocation

Membrane translocation of the toxins requires an acidified endosome. At acidic pH, the toxins undergo structural changes that lead to the exposure of hydrophobic domains and the subsequent insertion of the toxin into the endosomal membrane (Qa'Dan *et al.*, 2000); even a brief acidic pulse was found to be sufficient for insertion and increased permeability of cells to toxin (Barth *et al.*, 2001). The C-terminal of the toxin remains in the endosome, while the N-terminal reaches the cytosol (Pfeifer *et al.*, 2003). This receptor-mediated endocytosis is a dynamin-dependent process governed by clathrin (Fig. 1.3) (Papatheodorou *et al.*, 2010).

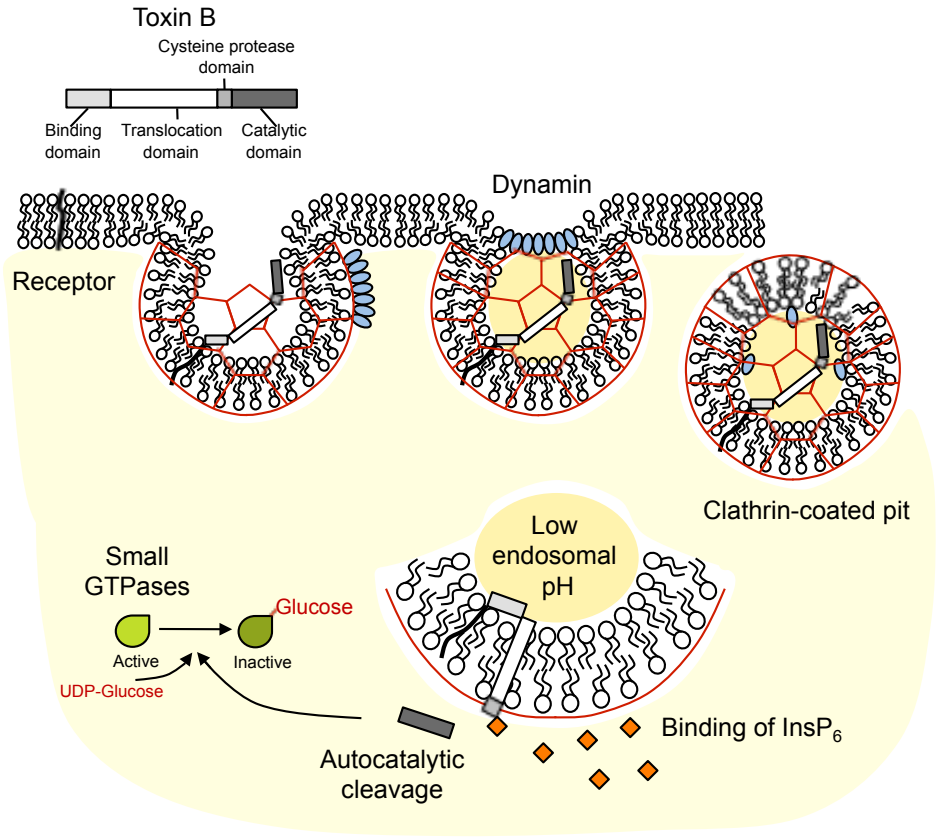
The N-terminal of TcdB was found to be capable of modifying Rho proteins, but was unable to intoxicate cells, suggesting pH-dependent proteolytic cleavage of the holotoxin during translocation into the cytosol (Pfeifer *et al.*, 2003). TcdB undergoes proteolytic cleavage between the Leu543 and Gly544 residues in VPI 10463 resulting in two fragments; one of 207 kDa fragment and the other of 63 kDa (Rupnik *et al.*, 2005). The 63 kDa fragment is the cytotoxic N-terminal of the holotoxin and is linked to the 207 kDa fragment by a peptide bond until translocation. The cleavage is dependent on the presence of the cytosolic factor, inositol hexakiphosphate ($InsP_6$), which causes a conformational change in the toxin (Reineke *et al.*, 2007). This $InsP_6$-dependent processing requires cysteine residues and therefore, occurs via a cysteine protease and not via an aspartate protease as suggested by Reineke and co-workers (Egerer *et al.*, 2007). The cysteine protease is composed of residues 544 to 955. The Lys600 residue is essential for $InsP_6$ to bind to the cysteine protease domain (Egerer *et al.*, 2009).

1.4.1.4. Effect on cells

Both TcdA and TcdB are capable of inducing rounding of epithelial cells and their detachment and removal from the basal lamina (Just *et al.*, 1994; Ketley *et al.*, 1987; Mahida *et al.*, 1996). This leads to acute mucosal inflammation, oedema, infiltration of polymorphonucleocytes and increased mucosal permeability (Ketley *et al.*, 1987; Savidge *et al.*, 2003). The toxins also induce apoptosis in cells such as epithelial

cells, T-cells and eosinophils (Fiorentini *et al.*, 1998; Mahida *et al.*, 1996; Mahida *et al.*, 1998). TcdB has been found to induce apoptosis in proliferating cells and necrosis in non-proliferating cells (Lica *et al.*, 2011). The characteristic cell rounding observed in TcdB-treated epithelial cells was found to be a result of the increased G- to F-actin ratio, suggesting an indirect catalytic effect on actin filaments owing to inactivation of Rho proteins (Huelsenbeck *et al.*, 2007; Just *et al.*, 1994; Pothoulakis *et al.*, 1986).

Fig. 1.3. Entry of *C. difficile* toxins into cells via clathrin-and dynamin-mediated endocytosis



The schematic model of the uptake and delivery of toxin B is represented. Receptor-mediated endocytosis is dependent on the presence of dynamin and controlled by clathrin. The acidic pH of the endosome triggers a conformational change in the toxin and results in pore formation in the endosomal membrane. Cytosolic $InsP_6$ then interacts with the toxin, which causes autocatalytic cleavage and the release of the catalytic glucosyltransferase domain into the cytosol, where it is able to act on the target cytosolic GTPases. This subsequently leads to the disruption of the actin cytoskeleton and cell death. Adapted from Papatheodorou *et al*., 2010 and Reineke *et al*., 2007.

1.4.1.5. Effect of glucosylation on GTPases

GTPases belong to a superfamily of low molecular weight proteins which act as molecular switches in key signalling pathways; they are involved in processes such as immune cell migration, epithelial barrier function, adhesion, progression of the cell-cycle, cytokine secretion, super-oxide production, phagocytosis and endocytosis (Aktories *et al.*, 2000). Rho GTPases occur in the cytosol in their inactive GDP-bound form and are associated with guanine nucleotide dissociation inhibitors (GDI); in their active form, they are membrane associated (Fig. 1.4) (Jank & Aktories, 2008; Schirmer & Aktories, 2004). Guanine nucleotide exchange factors (GEFs) activate GTPases and enables GTPases to interact with effectors to control cell signalling, while GTPase-activating proteins (GAP) inactivate GTPases by hydrolysis of bound GTP. *C. difficile* toxin A and toxin B act by the functional inactivation of GTPases.

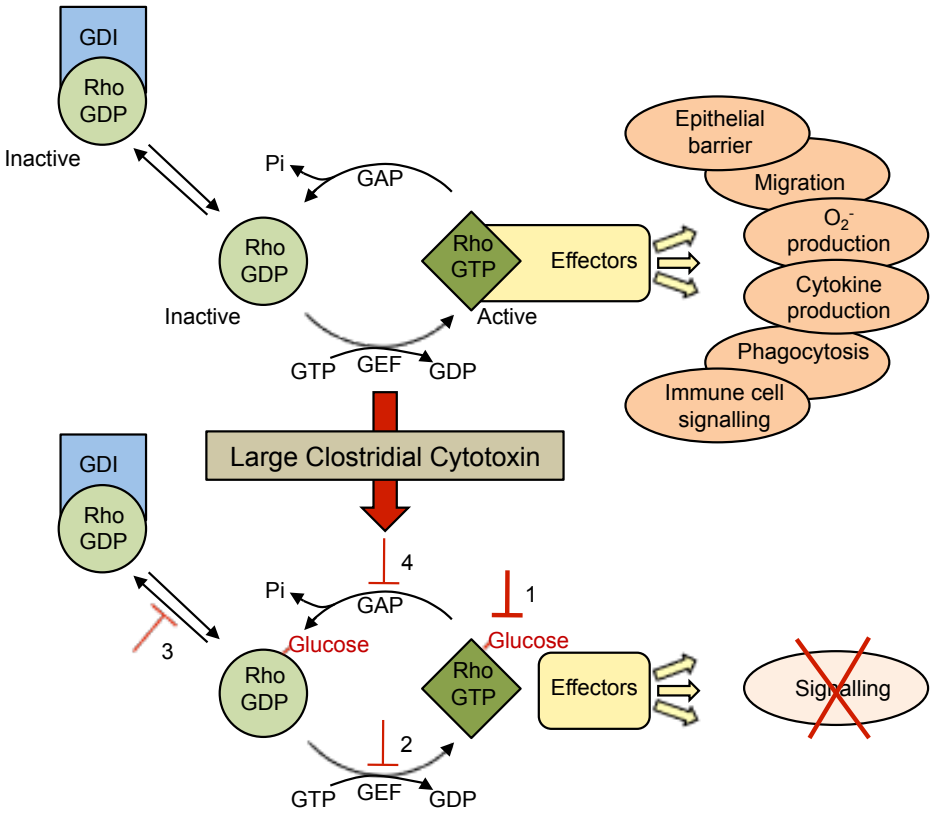
Preliminary studies showed that the toxins did not act on Rho via cGMP, cAMP or protein kinase C and therefore, acted directly on Rho and not on Rho-activating proteins (Just *et al.*, 1994; Just *et al.*, 1995a). Further investigations into the enzyme activity of the toxins revealed that the observed covalent modification of Rho was not a result of ADP-ribosylation or phosphorylation (Dillon *et al.*, 1995), but an uptake of one glucose molecule per molecule of Rho proteins (Just *et al.*, 1995b; Just *et al.*, 1995c). The toxins were thus confirmed to be monoglucosyltransferases that used UDP-glucose as the co-substrate. They were found to glucosylate RhoA/B/C at Thr37 and Rac and Cdc42 at Thr35 and recent evidence suggests that the glucosylation of Rac1 and not Rho that causes the observed cytopathic effects in intoxicated cells (Halabi-Cabezon *et al.*, 2008).

1.4.1.6. Immune response generated

The glucosylation of Rho proteins and subsequent actin depolymerisation in epithelial cells *in vivo* leads to cell-rounding and detachment from the basal membrane (Mahida *et al.*, 1996). The resulting lack of tight junctions causes increased colonic permeability, fluid accumulation and possible perforation, which may lead to watery diarrhoea (Lyerly *et al.*, 1982; Nusrat *et al.*, 2001). The following

acute inflammatory response involves the extravasation of large numbers of neutrophils, mast cells and macrophages and the release of pro-inflammatory cytokines like IL-1β, TNF-α and IL-8 (Kelly & Kyne, 2011; Savidge *et al.*, 2003). TcdA also induces enteric neurons to secrete substance P (Castagliuolo *et al.*, 1997) which activates mast cells, increasing mucosal secretion and inflammation (Wershil & Castagliuolo, 1998). Sloughed epithelial and immune cells and fibrin together constitute the resulting pseudomembrane (Knoop *et al.*, 1993; Linevsky *et al.*, 1997).

Fig. 1.4. Effect of *C. difficile* toxins on the GTPase cycle of Rho proteins



Rho, in its inactive form, is bound to GDP and associated with a guanine dissociation inhibitor (GDI). Activation of Rho proteins is catalysed by a guanine nucleotide exchange factor (GEF). Rho is active in its GTP-bound form and interacts with various effectors. Hydrolysis by a GTPase-activating protein (GAP) renders it inactive. Glucosylation by *C. difficile* toxin A and toxin B blocks the coupling of the Rho GTPases with effectors (1), which renders them functionally inactive. It also inhibits nucleotide exchange induced by GEFs (2), blocks the Rho/GDI interaction (3) and inhibits GTP hydrolysis stimulated by GAPs (4). Adapted from Schirmer & Aktories, 2004 and Jank & Aktories, 2008.

1.4.2. Binary toxin

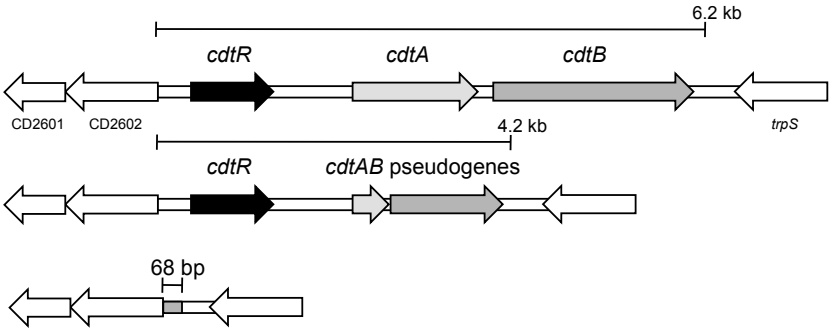
Some *C. difficile* strains also produce a binary toxin (CDT). Clostridial binary toxins are comprised of two molecules that are not linked through covalent or non-covalent bonds but both molecules are required for activity (Rupnik *et al.*, 2003a). This family of toxins includes *C. perfringens* type E iota toxin, *C. spiroforme* toxin and the C2 toxin from *C. botulinum* C and D.

CDT is coded on the CDTLoc, which consists of three genes: *cdtA*, which codes forthe enzymatic component, *cdtB*, which codes for the binding component, and the regulator, *cdtR* (Carter *et al.*, 2007; Perelle *et al.*, 1997). *cdtA* and *cdtB* show 81% and 84% identity with *iap* and *ibp* genes of the *C. perfringens* type E iota toxin, suggesting a common ancestor for these genes (Perelle *et al.*, 1997). *cdtA* is 1383 bp long and *cdtB* is 2631 bp long and are transcribed as a single mRNA (Rupnik *et al.*, 2003a). *cdtR* belongs to the *lyt*TR response regulator family (Carter *et al.*, 2007). In strains that do not produce the binary toxin, the CDTLoc is replaced by a 68 bp sequence and it is shortened in strains that contain pseudogenes for *cdtA* and *cdtB* (Fig. 1.5) (Carter *et al.*, 2007). The toxin consists of two subunits which are both produced and secreted and the toxin is activated by trypsinisation; CDTa is the enzymatic subunit and CDTb is the binding subunit which is responsible for the translocation of CDTa into the cytoplasm (Barth *et al.*, 2004; Perelle *et al.*, 1997).

CDT is an actin-specific ADP-ribosyltranferase that was first identified in 1988 (Popoff *et al.*, 1988). Originally, it was not found to be cytotoxic to tissue epithelial cells or lethal to animals on intraperitonial injection (Popoff *et al.*, 1988); however, cytotoxicity induced by it appears to be similar to that of toxin A and toxin B (Rupnik *et al.*, 2003a). The toxin acts by covalently modifying actin at the Arg177 residue (Popoff *et al.*, 1988; Schwan *et al.*, 2009). It inhibits F-actin formation and causes microfilament depolymerisation and tubule-formation (Schwan *et al.*, 2009). The induced cellular microtubule-based protrusions were found to increase the adherence of *C. difficile* cells to epithelial cells.

The role of CDT in the pathogenesis of CDI is still unknown, but its ability to contribute to disease has been demonstrated. The toxin was shown to induce fluid accumulation in the ligated loop model (Geric *et al.*, 2006). However, in the same study, infection of hamsters with a *C. difficile* strain which was A-B-CDT+ resulted in colonisation but no infection, suggesting that the binary toxin alone is not able to cause disease but can serve as an additional virulence factor for *C. difficile*. The presence of the binary toxin in *C. difficile* ribotype 027 has been suggested as a factor for its hypervirulence. This has been further discussed in Chapter 6 (6.1.4.2.2).

Fig. 1.5. CDTLoc of *C. difficile*



Schematic representation of the CDTLoc comprising *cdtA*, *cdtB* and *cdtR,* shows the complete 6.2 kb locus, the 4.2 kb locus in strains like *C. difficile* 630 that contain pseudogenes instead of *cdtA* and *cdtB* and the 68 bp sequence that replaces the CDTLoc in binary toxin-negative strains. The boundaries of the CDTLoc are conserved in all *C. difficile* strains. Adapted from Carter *et al.*, 2007.

1.4.3. Spores

C. difficile produces metabolically-dormant spores which represent the infectious stage of the bacterium (Wilson *et al.*, 1982). Spores play a role in the transmission, persistence and pathogenesis of *C. difficile*. It has been demonstrated that *C. difficile* spores can resist disinfection and persist in the environment for long periods of time. Mice can acquire these spores from the contaminated environment, asymptomatic carriage or infection can subsequently be induced and the antibiotic treatment could cause (super)shedding of spores through faeces of the infected animals excreted over extended periods of time, causing increased dissemination of spores and transmission

to susceptible individuals (Lawley *et al.*, 2009a; Lawley *et al.*, 2010). The sources of *C. difficile* spores and their survival, persistence and transmission are discussed in detail in Chapter 4 (4.1.1-4.1.5, 4.1.7).

Once ingested, *C. difficile* spores attach to the gastrointestinal tract via proteinaceous exosporial filaments (Panessa-Warren *et al.*, 1997). Although spores are highly infective, only vegetative cells of *C. difficile* produce the toxins that lead to infection (Sorg & Sonenshein, 2010). Thus, germination and outgrowth of spores is essential. Germination only occurs in nutrient-rich environments and is mediated via receptors on the spore surface (Foster & Johnstone, 1990; Setlow, 2003). These receptors are specific and use environmental conditions such as bile salt concentrations, pH and the presence of amino acids as cues for effective germination (Giel *et al.*, 2010; Howerton *et al.*, 2011). *C. difficile* spores have been shown to germinate in response to bile salts and taurocholate-mediated germination in the small intestine is most likely to occur in human infection (Howerton *et al.*, 2011; Lawley *et al.*, 2009b). Sodium taurocholate was found to act as a germinant only in the presence of glycine (Sorg & Sonenshein, 2008), while chenodeoxycholate and deoxycholate could facilitate germination, but were incapable of sustaining the growth of vegetative cells (Sorg & Sonenshein, 2008; Wheeldon *et al.*, 2008a). Contrary to these observations, it was found that glycine and taurocholate had no effect on germination but the presence of K^+, P_i and a pH of 6 enhanced the process (Paredes-Sabja *et al.*, 2008). Thus, *C. difficile* spores could also germinate in the duodenum at pH 6 or in the colon where the concentration of K^+ is high. Spore germination was reduced by acidic conditions and lower temperature, but not affected by aerobic conditions (Wheeldon *et al.*, 2008a).

The role of spores in pathogenesis is directly linked to the disruption of normal gut microbiota by antibiotics. Intestinal microbiota is able to modify primary bile salts like cholate and chenodeoxycholate to secondary bile salts like deoxycholate and lithocholate (Sorg & Sonenshein, 2008; Wilson, 1983). In the presence of chenodeoxycholate, spores show a reduced affinity for taurocholate and thus, there is

inhibition of sporulation (Sorg & Sonenshein, 2010). Further, the outgrowth of spores that do germinate is inhibited by deoxycholate (Giel *et al.*, 2010; Sorg & Sonenshein, 2010). Thus, in the presence of normal gut microbiota *C. difficile* spores are unable to successfully undergo germination and outgrowth. However, when the microbiota is perturbed there is a change in the ratio of primary to secondary bile salts due to the reduced numbers of bile salt-modifying bacteria, which makes conditions favourable for the germination and outgrowth of spores into toxin-producing vegetative cells (Giel *et al.*, 2010). This has been demonstrated in mice; mice treated with antibiotics have less diverse microbiota with fewer *Bacteroides* and *Firmicutes* (Giel *et al.*, 2010; Lawley *et al.*, 2009a) and their caecal contents stimulate significantly greater spore germination and outgrowth. Further, normal microbiota in mice was able to modify taurocholate and reduce its ability to induce germination. Although a precise germination factor has not yet been identified it is most likely a small, heat-stable, water-soluble bile salt present at high levels *in vivo* in response to antibiotics (Giel *et al.*, 2010).

Sporulation initiation in *C. difficile* is controlled by a two-component signal transduction system (TCS) in which environmental and cellular signals are sensed by sporulation-associated histidine kinases that phosphorylate Spo0A (Underwood *et al.*, 2009). The Spo0A response regulator is the master-regulator of sporulation in *Clostridium* and *Bacillus* species (Molle *et al.*, 2003). Spo0A and the gene encoding it, *spo0A*, are discussed later in Chapter 3 (3.1.5).

1.4.4. Surface-associated proteins

In order to establish an infection, pathogenic bacteria must normally be able to bind to host cells, colonise tissues, invade them and then persist or disseminate to other tissues while continually interacting with and modulating the immune system (Pizarro-Cerdá & Cossart, 2006). In *C. difficile*, a number of cell-surface associated virulence factors have been identified that act as adhesins and are also immunodominant (Péchiné *et al.*, 2005b). These virulence factors include the S-layer proteins, cell wall proteins (CWPs), flagella and heat-shock proteins (HSPs).

The S-layer of *C. difficile* is composed of two protein subunits that form a regularly arranged lattice and are essential for the structure of the cell wall (Cerquetti *et al.*, 2000; Sára & Sleytr, 2000). They are derived from a common precursor protein encoded by the *slpA* gene (Karjalainen *et al.*, 2001). The SLPs are involved in adherence to mucus and epithelial cells (Calabi *et al.*, 2002). They were also found to be the most commonly recognised antigens in CDI patients (Wright *et al.*, 2008). The flagella of *C. difficile* may also be involved in adherence; the flagellar filament, FliC, and the flagellar cap, FliD, are both able to mediate binding to mucus (Tasteyre *et al.*, 2001a). Antibody responses to both these antigens have been observed in patients (Péchiné *et al.*, 2005a). FliD appears to have a more important role in the initial attachment to mucus and this exposure to the immune system probably results in the detected higher antibody response to it as compared to FliC. The cell wall proteins Cwp66 and GroEL are both heat-shock proteins that are involved in adherence (Hennequin *et al.*, 2001b; Waligora *et al.*, 2001), and Cwp66 was found to be highly immunogenic in patients (Péchiné *et al.*, 2005a). These proteins are described in detail in Chapter 5 (5.1.1).

The surface-associated proteins Fbp68, Cwp84 and CwpV are also important virulence factors. Fbp68 is a fibronectin-binding protein present mainly in the cytoplasmic membrane of *C. difficile* cells (Hennequin *et al.*, 2003). It is able to bind to immobilised and soluble fibronectin and also fibrin, which can directly help in attachment to the surfaces of a variety of host cells and also indirectly aid binding through interactions of fibronectin with other structural proteins like collagen and fibrin. Fbp68 has also been found to elicit a high antibody response in patients (Péchiné *et al.*, 2005a).

Cwp84 is a cysteine protease containing the conserved Pept_C1 domain of the papain family (Savariau-Lacomme *et al.*, 2003). It can specifically degrade extracellular matrix (ECM) proteins such as fibronectin, laminin and vitronectin (Janoir *et al.*, 2007). This proteolytic degradation could allow *C. difficile* toxins to penetrate further into the basement membrane and also enhance the dissemination of

bacteria. Further, Cwp84 is involved in the maturation of the SLPs; it cleaves the SlpA precursor protein into its two subunits, but is not essential for this process (Kirby *et al.*, 2009). Due to its surface-association, Cwp84 is able to induce a very intense antibody response in patients (Péchiné *et al.*, 2005b).

CwpV is another surface-expressed putative adhesin in *C. difficile* (Emerson *et al.*, 2009). Just like the SLPs, it is also post-translationally modified into two subunits of 40 and 120 kDa, but Cwp84 is not involved in this maturation (Kirby *et al.*, 2009). The expression of *cwpV* is phase-variable and controlled by a site-specific recombinase (Emerson *et al.*, 2009). This variation may aid in evasion of the host immune system. Phase-variable expression of *C. difficile* flagella has also been suggested (Twine *et al.*, 2009). Thus, even though individually these surface-associated proteins are not responsible for specific functions, taken altogether, they participate in the attachment of the bacteria to the host, colonisation of the host and modulation of the immune response of the host.

The SLPs, Cwp66, Cwp84 and other similar proteins are encoded on the same cluster of the genome suggesting linked functions in maintenance of the cell wall in *C. difficile* (Calabi *et al.*, 2001; Calabi & Fairweather, 2002; Waligora *et al.*, 2001).

1.4.5. Others

The production of other virulence factors such as hyaluronidase, collagenase, protease and other hydrolytic enzymes that might contribute to adhesion and dissemination *in vivo* have also been identified in some *C. difficile* strains (Borriello *et al.*, 1990; Hafiz, 1974; Seddon *et al.*, 1990). In some strains, the presence of a capsule-like material has also been detected which could be involved in adhesion as well as evasion of the immune system of the host through its anti-phagocytic properties (Davies & Borriello, 1990). Rarely, the presence of fimbriae on the surface of *C. difficile* cells has also been identified, although their role in infection has been debated (Borriello *et al.*, 1990). Their absence does not appear to affect colonisation or infection (Borriello *et al.*, 1988; Taha *et al.*, 2007).

1.5. Aims

The main intention of this thesis was to identify inter-strain differences between historic and epidemic strains of *C. difficile*. Five strains of *C. difficile* were selected and used throughout this work. These were *C. difficile* strain 630, an historic isolate and the first strain to be sequenced, strain VPI 10463, a reference strain, ribotype 027, a hypervirulent epidemic isolate, and ribotypes 001 and 106, which are locally endemic in Scotland. A total of four studies were performed and their aims were as follows:

1. The first study was aimed at understanding the expression of the toxins and spores of the five *C. difficile* strains in a growth-dependent manner. This involved studying the phenotypes of the strains by measuring toxin production using immunoassays and spore production by viable counts. The expression of toxins and spores at the genetic level was analysed by studying the transcription of the five genes of the pathogenicity locus and *spo0A* using a real-time RT-PCR assay. The hypothesis for this study was that the hypervirulent epidemic strains exhibited greater transcription and release of both toxins and spores, which could result in the increased severity of disease often associated with them.

2. The second study was aimed at identifying the most suitable disinfectant against *C. difficile* for use in the laboratory from amongst five commonly-used agents, including one decontaminant. The susceptibility of the *C. difficile* strains to the agents was studied by conventional laboratory tests, followed by surface disinfection tests. The effects of organic matter on disinfection and on the exposure of spores to sub-inhibitory concentrations of the agents on toxin production were also studied. Further, contamination of the laboratory environment was also investigated. The hypothesis for this study was that the hypervirulent epidemic strains were more resistant to disinfection procedures and thus were able to survive longer in the environment. Further, the incorrect use of cleaning agents and the subsequent exposure of these strains to sub-inhibitory concentrations of the agents could affect their virulence.

3. The third study was aimed at examining the biological activities of the five *C. difficile* strains. This involved studying virulence factors of the strains such as S-layer proteins, heat-shock proteins and toxins for their ability to induce cytokine production by macrophages, and investigating the importance of flagella and S-layer proteins in adherence to epithelial cells. The hypothesis for this study was that the antigens of the epidemic strains would elicit a greater immune response within the host and thus, mediate greater damage. Further, their antigens would exhibit high adhesion to epithelial cells and thus, aid colonisation.

4. The fourth and final study was aimed at characterising a collection of ribotype 027 strains isolated in Scotland and another from the Netherlands. This involved typing the strains by several molecular methods, studying their phenotype, investigating their susceptibility to antibiotics and identifying genetic mutations that characterise them. Comparisons with the five strains used in the previous studies were also made. The hypothesis of this study was that there was a clonal spread of ribotype 027 strains.

2. Materials and Methods

2.1. Bacterial strains

2.1.1. Reference strains

Throughout this work, two reference strains were used. These were *C. difficile* strain 630, which belongs to ribotype 012 and also represents an historic isolate found in Scotland, and strain VPI 10463, a known high toxin producer commonly used as a reference strain in toxin production studies.

2.1.2. Epidemic strains

The three *C. difficile* strains selected for comparison in growth-related, immune response and disinfectant studies were representative of the most commonly isolated PCR ribotypes in Scotland - ribotype 106, ribotype 001 and the hypervirulent ribotype 027.

2.1.3. Hypervirulent strains

Seven isolates of PCR ribotype 027 from Scotland and five isolates from the Netherlands were compared using different phenotypic and genotypic methods to identify clonal expansion of this ribotype in a small study. The isolates and their sources are listed in Table 2.1.

2.1.4. Clinical isolates

Twenty-five clinical isolates of different ribotypes and antibiotic sensitivity patterns (Mutlu *et al.*, 2007) were selected for disinfectant sensitivity assays. They are listed in Table 2.2.

2.2. Phenotypic characteristics

2.2.1. Growth

C. difficile strains were cultured from freeze-dried stocks by re-suspending the cells in 200 µl anaerobe identification medium (AIM; 20 g/L proteose peptone, 5 g/L yeast

extract, 5 g/L trypticase, 5 g/L NaCl, 0.75 g/L cysteine-HCl, 0.4 g/L Na_2CO_3; pH 7.1) (Brown *et al.*, 1996), followed by plating on blood agar (39 g/L Columbia blood agar base, 5% defibrinated horse blood) which were incubated anaerobically (80% N_2, 10% H_2, 10% CO_2) at 37°C in a Mark III workstation (Don Whitley Scientific) for 48 h. The strains were subcultured again on blood agar. The purity of the strains was checked by Gram-staining and the purified strains were stored as spores in Robertson's cooked medium (Watt, 1973) at room temperature.

Table 2.1. Isolates of PCR ribotype 027 and their sources

Isolates from Scotland		Isolates from the Netherlands	
MPRL Number	Source	MPRL Number	Source
4863	Glasgow Western	4828	Haarlem Verpl
4864	Glasgow Western	4829	Amersfoort
4865	Glasgow Victoria	4830	Haarlem
4866	Glasgow Western	4831	Amsterdam
4867	Dundee Ninewells	4832	Harderwijk
4868	Glasgow Victoria		
4883	Edinburgh Royal Infirmary		

2.2.2. Starter cultures

To grow *C. difficile*, starter cultures were always prepared by inoculating 0.5 ml of the purified spore suspensions into 3 ml of pre-reduced AIM and incubating them anaerobically for 16 h at 37°C till an OD_{600} of 1.0 (±0.05) was achieved. The appropriate volume of the starter culture was then inoculated into pre-reduced AIM to give a 1% culture, which was grown anaerobically at 37°C for 24 h. The purity of the cultures was checked by Gram-staining and also by aerobic and anaerobic subculture on blood agar.

Table 2.2. Clinical isolates used in this study and their antibiotic sensitivity profiles

Strain no.	Ribotype	Antibiotic sensitivity profile [a]				
		erythromycin	clindamycin	ceftriaxone	tetracycline	moxifloxacin
1	106	R	R	R	S	R
2	106	R	R	R	S	R
4	023	S	S	R	S	S
5	014	R	R	S	S	S
6	042	R	S	S	S	S
8	106	R	R	R	S	R
9	002	S	R	R	S	S
10	070	S	S	S	S	S
11	005	S	S	S	S	S
12	049	S	S	S	R	S
13	014	R	R	R	S	R
14	002	R	R	S	S	S
15	005	R	R	S	S	S
16	106	R	R	R	S	S
17	171	**	**	**	**	**
18	126	**	**	**	**	**
19	013	**	**	**	**	**
20	001	R	R	R	S	R
21	020	R	R	R	S	S
22	001	R	R	R	S	R
23	001	R	R	R	R	R
24	013	R	R	R	R	S
25	**	R	R	R	S	R

[a] All the isolates were sensitive to metronidazole and vancomycin.

** Undetermined

2.2.3. Growth curves

To study the growth curves of the strains, 3 ml of the starter culture was inoculated into 300 ml pre-reduced AIM. The growth of the strains was studied over 24 h by measuring OD_{600}. At 0, 4, 8, 12, 16, 20 and 24 h, the culture was gently mixed and 1 ml of the culture was transferred to a cuvette inside the anaerobic workstation, sealed and then removed to the CE 2021 spectrophotometer (Cecil Instruments) to measure OD_{600} compared to a medium blank. The growth curves were performed in triplicate. In some studies, viable counts were also performed. At every 4 h, 100 µl culture was removed and serially diluted 10-fold in pre-reduced AIM. At every time-point, 100 µl of three selected dilutions were spread onto blood agar plates and incubated at 37°C for 24 h. The plates were counted and the number of cells/ml culture was determined. To study the growth more closely, a 0.1% culture was used and OD_{600} of the culture was measured every hour up to 8 h and then every 24 h from 24 h up to 10 d.

2.2.4. Toxin production

Total toxin (A+B) production was studied using the *C. difficile* TOX A/B II™ kit (Techlab). Supernatants were collected by centrifuging 1 ml of culture at 16000 g for 1 min. They were diluted 5-fold in buffered diluent. The wells provided with the kit were coated with affinity purified goat antibodies specific for toxin A and toxin B. Two wells were prepared for each sample by adding one drop (50 µl) of the conjugate containing two horseradish peroxidase coupled antibodies - a mouse monoclonal antibody specific for toxin A and a goat polyclonal antibody specific for toxin B. Sample (100 µl) was added to each well. For the positive controls, one drop of the provided positive control was added per well and for the negative controls, one drop of diluent was added. The wells were sealed, incubated at 37°C for 50 min and washed 5 times with the provided wash solution composed of PBS and a detergent. Two drops (100 µl) of substrate (tetramethylbenzidine and peroxide) was then added to the wells and they were incubated at room temperature for 10 min with constant shaking. The reaction was stopped by adding one drop (50 µl) of the stop solution (0.3 M sulphuric acid) to each well. After incubating the plates for 2 min at room

temperature, the intensity of the colour developed was determined by measuring absorbance at 450 nm with the subtraction of absorbance at 620 nm ($A_{450/620}$). To measure the individual production of toxin A and toxin B, ELISAs were performed as described later (2.5.4). Culture supernatants were collected at the different time-points, aliquoted and stored at -70°C until use; samples were only thawed once.

2.2.5. Spore production

Spore production was assessed by viable counts. At selected time-points, 10 ml of the culture was collected by centrifugation at 4000 g for 10 min. The cell pellets were washed twice in distilled water and re-suspended in 50% ethanol. They were incubated at room temperature for 1 h with constant shaking. The cells were collected by centrifugation and washed twice with distilled water. The final cell pellet containing the alcohol-resistant spores was re-suspended in 1 ml distilled water. Ten-fold dilutions of this suspension were prepared in distilled water, spread plated on blood agar and incubated anaerobically for 48 h. The number of spores/ml culture was determined from the colony counts obtained.

2.2.6. Motility assay

C. difficile was cultured anaerobically at 37°C on blood agar for 24 h. Three colonies were picked up from the plate and inoculated into the top 5 mm of a tube containing 25 ml pre-reduced 0.05% brain heart infusion (BHI; Fluka) agar. The cultures were incubated anaerobically overnight (approximately 16 h). Growth was observed and the distance travelled from the zone of inoculation was recorded in centimetres (cm).

2.2.7. Autoagglutination assay

C. difficile was cultured anaerobically at 37°C on blood agar for 24 h. Five colonies were suspended in pre-reduced PBS, pH 7.2, to OD_{600} of 1.00 (±0.05). The suspension was incubated anaerobically for 24 h at 37°C, after which the OD_{600} was measured again. The autoagglutination was calculated as follows:

$$\% \text{ autoagglutination} = [(\text{starting } OD_{600} - \text{final } OD_{600}) / \text{starting } OD_{600}] \times 100$$

2.2.8. S-layer typing

C. difficile was cultured anaerobically (50 ml) at 37°C in AIM overnight (approximately 16 h). Surface-layer proteins (SLPs) were extracted from the culture as described later (2.4.4). The high molecular weight and low molecular weight SLP subunits were separated on an SDS-PAGE gel (2.4.7). The molecular weights of the subunits were determined using the protein marker on the gel and the Phoretix-1D software (TotalLab). S-layer types were assigned using these values.

2.3. Genotypic studies

2.3.1.1. DNA extraction

Genomic DNA (gDNA) was extracted from 1 ml of an overnight culture of *C. difficile* collected by centrifugation at 16000 g for 2 min. When using the NucleoSpin® kit (Macherey-Nagel GmbH), cells were lysed in a proteinase K/SDS solution at 56°C for 3 h. DNA in the lysate was bound to a silica filter by the addition of ethanol. After washing with two buffers to remove contaminants, DNA was eluted under low ionic strength using 100 µl of alkaline elution buffer. Alternately, cells were boiled in 100 µl of a 5% suspension of Chelex®100 (BioRad) prepared in DEPC water (Ambion) for 10 min to destroy the cell membranes and proteins and denature the gDNA. The suspension was centrifuged at 16000 g for 2 min to separate the resin and cell debris from the supernatant containing DNA. The concentration of DNA was determined using the ND-1000 spectrophotometer (Nano Drop Technologies), using the elution solution as the blank. The DNA was aliquoted and stored at -20°C.

2.3.2. Ribotyping

Amplification of the 16S-23S rRNA intergenic spacer region was performed as previously described (O'Neill *et al.*, 1996). The primers used were:

5’-CTGGGGTGAAGTCGTAACAAGG-3’
5’-GCGCCCTTTGTAGCTTGACC-3’

Reactions (50 µl) were set up containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 2 U of Taq polymerase (Promega), 0.2 mM of each dNTP, 50 pmol of each primer and 10 µl gDNA. The thermal profile used for the PCR was as follows:

Initial denaturation	94°C	3 min
35 amplification cycles:		
Denaturation	96°C	1 min
Annealing	56°C	1 min
Final extension	72°C	5 min

The PCR products were concentrated by heating at 75°C for 45 min. Electrophoresis was performed in a 3% Metaphor agarose gel (Cambrex Bio Science) containing 30 µl of SafeView Nucleic Acid Stain (NBS Biologicals) at 80 V for 3 h along with a 100 bp ladder (Promega). The gels were photographed under UV light and the data were analysed using the Gel Compar software (Bionumerics).

2.3.3. Toxinotyping

Toxinotyping was performed as previously described (Rupnik *et al.*, 1997). The first 3 kb of *tcdB* (PCR fragment B1) and 3 kb of the repetitive region of *tcdA* (PCR fragment A3) were amplified by PCR. This was followed by restriction fragment length polymorphism (RFLP) analysis of both the amplified products. The primers used to amplify the B1 fragment were:

5’-AGAAAATTTTATGAGTTTAGTTAATAGAAA-3’
5’-CAGATAATGTAGGAAGTAAGTCTATAG-3’

The primers used to amplify the A3 fragment were:

5’-TATTGATAGCACCTGATTTATATACAAG-3’
5’-TTATCAAACATATATTTTAGCCATATATC-3’

PCR reactions (50 µl) were set up containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 0.5 U of Taq polymerase, 0.2 mM of each dNTP, 50 pmol of each primer

and 5 µl gDNA. For amplification of A3, 5 mg of bovine serum albumin (BSA, Sigma-Aldrich), 0.05% W1 and TMA (tetramethylammonium chloride, Sigma-Aldrich) at a final concentration of 1 mM were also added to the reaction. The thermal profile used was:

Initial denaturation 93°C 3 min

35 amplification cycles:

Annealing/Extension 47°C 8 min

Denaturation 93°C 4 s

Final extension 47°C 10 min

PCR products were concentrated by heating at 75°C for 45 min and RFLP was performed at 37°C for 2 h. For the A3 fragment, 10 µl of the product was digested with 2 µl of *Eco*RI. For the B1 fragment, two digestions were performed with *Acc*I and *Hin*dI in which 2 µl of the product was digested with 2 µl of enzyme.

2.3.4. Binary toxin detection

The binary toxin genes, *cdtA* and *cdtB*, were detected as previously described (Stubbs *et al.*, 2000). The primers used to amplify *cdtA* were:

5'-TGAACCTGGAAAAGGTGATG-3'

5'-AGGATTATTTACTGGACCATTTG-3'

The primers used to amplify *cdtB* were:

5'-TTAATGCAAGTAAATACTGAG-3'

5'-AACGGATCTCTTGCTTCAGTC-3'

PCR reactions (50 µl) were set up containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 1 U of Taq polymerase, 0.2 mM of each dNTP, 50 pmol of each primer and 5 µl gDNA. The thermal profile used was:

Initial denaturation 94°C 5 min

35 amplification cycles:

Denaturation	94°C	30 s
Annealing	52°C	30 s
Extension	72°C	1 min
Final extension	72°C	5 min

2.3.5. Flagellum analysis

fliC and *fliD* were amplified as previously described. The primers used for *fliC* (Tasteyre *et al.*, 2000b) were:

5'-ATGAGAGTTAATACAAATGTAAGTGC-3'
5'-CTATCCTAATAATTGTAAAACTCC-3'

The primers used to amplify *fliD* (Tasteyre *et al.*, 2001b) were:

5'-ATGTCAAGTATAAGTCCAGTAAG-3'
5'-TTAATTACCTTGTGCTTGTG-3'

PCR reactions (50 µl) were set up with 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 1 U of Taq polymerase, 0.2 mM of each dNTP, 50 pmol of each primer and 5 µl gDNA. The thermal profile used was:

Initial denaturation	94°C	5 min

35 amplification cycles:

Denaturation	94°C	30 s
Annealing	55°C	30 s
Extension	72°C	1 min
Final extension	72°C	10 min

RFLP was performed at 37°C for 30 min. The reactions (20 µl) were set up with 2 µl of the product and 2 µl of the restriction enzyme. *fliC* products were digested with *Rsa*I, *Hin*dIII and *Hpa*I and *fliD* products were digested with *Acc*I, *Dra*I, *Eco*RI, *Hpa*I, *Hin*cII, *Hin*dIII, *Rsa*I and *Xba*I.

2.3.6. PCR amplification of *tcdC*

tcdC was amplified as described (Spigaglia & Mastrantonio, 2002) using the primers:

5'-TTAATTAATTTTCTCTACAGCTATCC-3'
5'-TCTAATAAAAGGGAGATTGTATTATG-3'

Reactions (50 µl) were set up with 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 1 U of Taq polymerase, 0.2 mM of each dNTP, 50 pmol of each primer and 5 µl gDNA. The thermal profile used was:

Initial denaturation	94°C	5 min

30 amplification cycles:

Denaturation	95°C	1 min
Annealing	52°C	30 s
Extension	72°C	1 min
Final extension	72°C	10 min

2.3.7. PCR amplification of *tcdR*

tcdR was amplified as described (Spigaglia & Mastrantonio, 2004) using the primers:

5'-CTCAGTAGATGATTTGCAAGAA-3'

5'-TTTTAAATGCTCTATTTTTAGCC-3'

Reactions (50 µl) were set up with 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 1 U of Taq polymerase, 0.2 mM of each dNTP, 50 pmol of each primer and 5 µl gDNA. The thermal profile used was:

Initial denaturation	94°C	5 min

30 amplification cycles:

Denaturation	94°C	1 min
Annealing	50°C	1 min
Extension	72°C	1 min

Final extension	72°C	10 min

2.3.8. PCR amplification of *tcdE*

A region of the *C. difficile* genome containing *tcdE* was amplified as previously described (Tan *et al.*, 2001) using the following primers:

5'-CGCGGATCCATGCACAGTAGTTCACCTT-3'
5'-CCCCCAAGCTTCCAACTGACCATGCACC-3'

Reactions (50 µl) were set up with 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 1 U of Taq polymerase, 0.2 mM of each dNTP, 50 pmol of each primer and 5 µl gDNA. The thermal profile used was:

Initial denaturation	94°C	2 min
Annealing	49°C	45 s
Extension	72°C	2 min

35 amplification cycles:

Denaturation	94°C	30 s
Annealing	52°C	30 s
Extension	72°C	50 s
Final extension	72°C	2 min

2.3.9. PCR amplification of *gyrA* and *gyrB*

gyrA and *gyrB* were amplified as described (Drudy *et al.*, 2006). The primers used to amplify *gyrA* were:

5'-TTGAAATAGCGGAAGAAATGA-3'
5'-TTGCAGCTGTAGGGAAATC-3'

The primers used to amplify *gyrB* were:

5'-GAAGGTCAAACTAAAACAAA-3'
5'-GGGCTCCATCTACATCAG-3'

PCR reactions (50 µl) were set up containing 1.5 mM MgCl2, 10 mM Tris-HCl, 50 mM KCl, 1 U of Taq polymerase, 0.2 mM of each dNTP, 50 pmol of each primer and 5 µl gDNA. The thermal profile used was:

Initial denaturation	95°C	3 min

30 amplification cycles:

Denaturation	95°C	1 min
Annealing	46°C	1 min
Extension	72°C	1 min
Final extension	72°C	10 min

2.3.10. PCR amplification of *slpA*

slpA was amplified as described (Karjalainen *et al.*, 2002). For amplification of the whole gene, the primers used were:

5'-ATGAATAAGAAAAACTATTAGCAATAGGC-3'
5'-AGCTGATACCTTTACCATACTTG-3'

To amplify the variable region, the primers used were:

5'-ATGAATAAGAAAAACTATTAGCAATAGGC-3'
5'-TCTATTCTATCTTCTCCATGCTAC-3'

PCRs were performed in 50 µl reactions containing 1.5 mM MgCl2, 10 mM Tris-HCl, 50 mM KCl, 1 U of Taq polymerase, 0.2 mM of each dNTP, 50 pmol of each primer and 5 µl gDNA. The thermal profile used was:

Initial denaturation	95°C	5 min

35 amplification cycles:

Denaturation	95°C	30 s
Annealing	45°C	1 min
Extension	72°C	2 min

Final extension 72°C 10 min

2.3.11. Electrophoresis

PCR products were loaded onto a 1.5% agarose gel (Sigma-Aldrich) prepared in Tris-borate-EDTA buffer (TBE) containing 10 µl of SafeView nucleic acid stain (NBS Biologicals) along with a 100 bp or 1 kb ladder (Promega) depending on the PCR, to determine the product size. Electrophoresis was carried out at 100 V for 100 min. The products were visualised under UV light.

2.3.12. Gene sequencing

C. difficile genes were sequenced to identify genomic differences between them. The genes were amplified by PCR and the products were visualised on a 1.5% agarose gel. Once the size and purity of the products was confirmed, they were cleaned of dNTPs and phosphoric groups by treatment with Antartic phosphatase (New England BioLabs) at 37°C for 15 min followed by inactivation of the enzyme at 80°C for 15 min. Two reactions were set up for sequencing in a BigDye reaction - one with the forward primer and one with the reverse primer, both at 3.2 pmol. The generated sequences were analysed using the Chromas Lite software (http://www.technelysium.com.au/chromas_lite.html) and aligned using the MultAlin software (Corpet, 1988). Further, the protein sequences were obtained using the ExPASy Translate tool (Gasteiger *et al.*, 2003) (the Swiss Institute of Bioinformatics (SIB); http://www.expasy.ch/tools/dna.html) and compared using MultAlin.

2.4. Extraction of Antigens

2.4.1. Dialysis culture of *C. difficile*

C. difficile strain VPI 10463 was used to obtain toxin A and toxin B as it is a known high toxin producer based on a dialysis culture method (Kamiya *et al.*, 1988). Starter cultures were prepared as described in 2.2.2. Dialysis tubing with a molecular weight cut-off (MWCO) of 10,000 Da was prepared by boiling twice in distilled water for 10 min. Once cooled, the tubing was sealed at one end and attached to the ventilated

lid of a culture bottle containing 400 ml AIM. The tubing was filled with 100 ml sterile saline (0.85% NaCl). The apparatus was autoclaved and placed under anaerobic conditions overnight. Starter culture was added to the dialysis bag and the culture was allowed to grow anaerobically at 37°C for 5 d. While medium components could diffuse into the dialysis tubing to support bacterial growth and small proteins could diffuse out of the tubing, toxin A and toxin B which are larger than the MWCO of the tubing, were contained within it. After 5 d, the culture was harvested by centrifugation at 5000 g for 20 min. The supernatant was dialysed against TBS (0.02 M Tris-HCl, 0.5 M NaCl; pH 7.5) at 4°C overnight, filtered through a 0.22 µm filter and stored at 4°C.

2.4.2. Purification of toxins by affinity chromatography

Toxin A was purified by affinity chromatography (Kamiya *et al.*, 1989; Krivan & Wilkins, 1987). Bovine thyroglobulin (500 mg, Sigma-Aldrich) was dissolved in 100 ml 0.1 M MOPS (morpholinepropanesulfonic acid; pH 7.0) that had been clarified by centrifugation at 8000 g and filtered through a 0.22 µm filter. CNBr-activated Sepharose gel (4.4 g; Amersham Biosciences) was washed thoroughly in 1 mM HCl to remove any additives and was allowed to react overnight at 4°C with 60 ml of the bovine thyroglobulin prepared. The mixture was blocked with 40 ml 0.1 M ethanolamine at 4°C for 30 min and washed in 0.1 M MOPS buffer. The coupled beads were packed into a column (C10; Amersham Biosciences) and washed at 37°C with 120 ml pre-warmed basic buffer (0.1 M glycine-NaOH with 0.5 M NaCl; pH 10.0) and 120 ml pre-warmed acidic buffer (0.1 M glycine-HCl with 0.5 M NaCl; pH 2.0). The column was equilibrated at 4°C with 120 ml TBS. Dialysed filtrate from above was applied to the column. The flow through containing toxin B was collected and toxin A bound to the column was eluted with 50 ml TBS at 37°C. Absorbance of the fractions was measured at 280 nm. The fractions were pooled, sterilised through a 0.22 µm filter and stored at 4°C. To reuse the column, it was washed with 60 ml acidic buffer at room temperature and equilibrated with 60 ml TBS at 4°C. The presence of toxins was determined using the *C. difficile* Tox A/B II™ kit.

2.4.3. Purification of toxins by ammonium sulphate precipitation

C. difficile toxins were prepared by ammonium sulphate precipitation (Pothoulakis *et al.*, 1986). The pH of the dialysed filtrate containing toxins was adjusted to 7.2 by the addition of solid Tris base. Ammonium sulphate was slowly added to the filtrate with constant stirring at 4°C until it was 70% saturated. After stirring for 1 h at 4°C, precipitated toxin B was collected by centrifugation at 3000 g for 15 min and re-suspended in PBS. The supernatant was then saturated to 20% with ammonium sulphate. After stirring for 1 h, the supernatant was collected by centrifugation, saturated to 50% and incubated for a further 1 h with constant stirring. Precipitated toxin A was collected by centrifugation at 3000 g for 15 min and re-suspended in PBS. The crude toxins were dialysed against PBS overnight with three changes of PBS. The presence of toxins was confirmed using the *C. difficile* Tox A/B II™ kit.

2.4.4. Preparation of S-layer proteins

Overnight cultures (50 ml) of *C. difficile* were harvested by centrifugation at 4000 g for 20 min. The cell pellets obtained were washed twice in 10 ml PBS, re-suspended in 3.75 ml of 5 M guanidine hydrochloride (GHCl) and incubated at room temperature for 2 h with constant shaking for extraction of S-layer proteins. The cell debris was separated from the supernatant containing the SLPs by centrifugation at 4000 g for 20 min. The supernatant was dialysed against PBS for 24 h with three changes of PBS. The dialysed protein was collected, aliquoted and stored at -20°C.

2.4.5. Preparation of flagella

Overnight cultures (1 L) of *C. difficile* were harvested by centrifugation at 13000 g for 10 min at 4°C. The cell pellets obtained were washed once in 500 ml PBS, re-suspended in 20 ml PBS and left overnight at 4°C. The cells were homogenised at full speed in a Waring blender for 2 min and centrifuged at 12000 g for 10 min at 4°C. The supernatant was centrifuged at 12000 g for 10 min at 4°C to remove cell debris. The supernatant was then centrifuged at 25000 g for 1 h at 4°C to collect the flagella. The pellets were re-suspended in 1 ml PBS, aliquoted and stored at -20°C.

2.4.6. Preparation of heat-shock proteins

C. difficile was grown till the culture reached an OD_{600} of 0.5-0.7 and divided into 3 aliquots of 25 ml. The aliquots were incubated at different temperatures for 30 min excluding the time taken to reach the optimum temperatures of 42°C for expression of GroEL, 60°C for expression of Cwp66 and 37°C for the non-shock control. Immediately after the heat treatment, the cultures were collected by centrifugation at 4000 g for 20 min. The cells were lysed at 37°C in a sonicating water bath for 5 min to release the heat-shock proteins. The cells were pelleted by centrifugation at 16000 g for 2 min and the supernatants were collected, aliquoted and stored at -20°C.

2.4.7. SDS-PAGE

Denaturing SDS-PAGE was performed in a vertical minigel apparatus (Bio-Rad). The gels were prepared by combining pre-determined volumes of 40% acrylamide solution, Tris-HCl buffers, SDS solution, ammonium persulphate solution (APS) and TEMED in distilled water to obtain a 4% stacking gel (prepared in 0.75 M Tris-HCl; pH 6.8, 0.2% SDS) and a 12% resolving gel (prepared in 0.25 M Tris-HCl; pH 8.8, 0.2% SDS). The samples were prepared by boiling 10 µl of protein with 10 µl of a double-strength sample buffer (0.125 M Tris-HCl; pH 6.8, 4% SDS, 20% glycerol, 2% 2-mercaptopethanol, 0.002% bromophenol blue) at 100°C for 3 min. The samples were loaded on the gel in 10 µl volumes with 6 µl of Mark 12™ Unstained Protein Standard (Invitrogen). Electrophoresis was performed in a 0.5 M Tris-glycine buffer with 0.1% SDS at 150 V for 1.5 h. The gels were washed once in distilled water for 5 min and stained with colloidal Coomassie blue stain G250 (Severn Biotech) for 1 h. This was followed by washing in distilled water for 1 h and then overnight for complete destaining. The gels were visualised under epi-white light.

2.4.8. Bradford assay

The concentration of toxins was determined by the Bradford assay (Bradford, 1976). Bradford reagent (Sigma-Aldrich) was used and the assay was performed according to the manufacturer's instructions for a 96-well plate assay protocol. Two-fold dilutions of BSA (Sigma-Aldrich) were prepared in DEPC water (Ambion) ranging

from 2 mg/ml to 0.125 mg/ml as protein standards. The standards and samples (5 µl) were added to the wells of a 96-well plate in duplicate, followed by the addition of 250 µl of Bradford reagent at room temperature. Negative controls were maintained with PBS. The plates were placed on a shaker for 30 s, followed by incubation at room temperature for 30 min, following which absorbance at 595 nm (A_{595}) was measured. The A_{595} values of standard BSA dilutions were plotted against concentration to generate a standard curve, from which protein concentrations of the samples were extrapolated using the GraphPad Software Prism 4.0.

2.4.9. Limulus amoebocyte lysate (LAL) assay

An end-point LAL assay was performed to detect LPS contamination in the antigens using the Pyrochrome® reagent (Associates of Cape Cod) as per the manufacturer's instructions. The reagent was re-suspended in 3.2 ml of buffer and kept on ice until use. To prepare the standard, 0.2 ng *E. coli* endotoxin was re-suspended in 4 ml LAL reagent water (LRW) to obtain a concentration of 0.5 endotoxic units per millilitre (EU/ml). Two-fold dilutions of standard were used to generate standard curves. Antigens were diluted in PBS and 50 µl was added to two wells in a 96-well plate. Negative controls with PBS were maintained. The Pyrochrome® reagent (50 µl) was added to each well and the plate was incubated at 37ºC for 30 min. The reaction was stopped by adding 25 µl of 50% acetic acid and A_{405} was measured.

2.4.10. Silver staining

The antigens were run on an SDS-PAGE gel with an LPS control. For silver staining, the gel was covered with fixative (25% propan-2-ol, 7% acetic acid) and incubated overnight to fix any LPS to the gel. The fixative was discarded and the gel was oxidised in 154 ml oxidiser (0.7% periodic acid in fixative) for 15 min. The gel was washed in distilled water four times for 1 h. Freshly-prepared ammoniacal silver nitrate solution (0.076% NaOH, 0.014% ammonia solution, 0.078% silver nitrate solution) was added to the gel for 15 min. The gel was washed four times in distilled water and incubated with freshly-prepared developer (0.005% citric acid in 0.019%

formaldehyde) until optimal staining intensity was achieved. After washing in distilled water twice for 10 min, the gel was visualised under epi-white light.

2.5. Immunoassays

2.5.1. Dot blots

Dot blots were performed for antigen detection. Squares of approximately 1 cm^2 were marked on a nitrocellulose membrane (BioTrace NT; Gelman Sciences) in pencil. The membrane was washed in PBS for 10 min and dried at room temperature. Samples were inoculated onto the nitrocellulose membrane by allowing 2 µl of the sample to diffuse onto the centre of each square. The membrane was dried completely by incubating at room temperature first for 10 min and then at 37°C for 5 min. The membrane was subsequently washed twice in PBS-Tween for 10 min and blocked with 3% PBS-gelatin for 45 min at 37°C. The blocking solution was replaced with 5 ml of a suitable dilution of primary antibody prepared in 1% PBS-gelatin and the membrane was incubated at 37°C for 3 h. After washing twice in 0.05% PBS-Tween, 5 ml of a suitable dilution of the detection antibody prepared in 1% PBS-gelatin was added and the membrane was incubated at 37°C for 1 h. The membrane was washed thoroughly in 0.05% PBS-Tween and then in three washes of distilled water to remove any residual Tween 20. The horseradish peroxidase (HRP) colour development solution (BioRad) was prepared by mixing freshly-prepared solution A (60 mg 4-chloro-1-naphthol dissolved in 20 ml methanol) with solution B (60 µl hydrogen peroxide added to 100 ml PBS). This development solution was added to the membrane and it was incubated at room temperature in the dark with constant shaking for up to 45 min until the purple colour of the reaction became visible. The reaction was stopped by washing the membrane in several washes of distilled water. The membrane was dried and stored at room temperature.

2.5.2. Western blots

For western blotting, antigens were run on an SDS-PAGE gel along with a ColorPlus prestained protein marker (New England Biolabs). The nitrocellulose membrane was

immersed in transfer buffer (0.025 M Tris, 0.192 M glycine; pH 8.3, 20% methanol) for 10 min. The gel was washed in distilled water and also placed in the transfer buffer for 10 min. The western blot cassette was set up with the gel and the nitrocellulose membrane in contact. Antigens were transferred from the gel onto the membrane at 5 V overnight at 4°C. The membrane was washed in PBS-Tween for 10 min, blocked, treated with antibodies and developed as described in 2.5.1.

2.5.3. Protein quantification from dot blots

For the quantification of proteins, images of the nitrocellulose membranes were analysed using Adobe Photoshop CS. The method was first standardised with dot blots performed with lipopolysaccharide (LPS). LPS extracted from *E. coli* R1 was dissolved in the 0.2% triethylamine (TEA). Two-fold dilutions of the LPS, ranging from 500 ng/ml to 15.125 ng/ml, were prepared in distilled water. The dot blots were performed as described in 2.5.1 with WN1 222-5 monoclonal antibody. After the dot blot was completed, the nitrocellulose was photographed and the image was stored as a TIFF file. Using Adobe Photoshop CS, areas were selected over the centre of each dot with the same numbers of pixels and the intensity of the pixels was obtained from the histogram generated. The intensity of the background was subtracted from these values and the values were plotted against the protein concentration to generate a standard curve from which the concentration of protein in the unknown sample could be determined.

2.5.4. ELISA procedure

ELISAs were developed for the separate quantification of *C. difficile* toxin A and toxin B as well as for seven cytokines. In general, the primary antibody was diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6 and coated onto a Nunc MaxiSorp® flat-bottom 96-well plate and incubated overnight at 4°C. The plate was washed thoroughly with 0.05% PBS-Tween and blocked with 100 µl 3% PBS-gelatin for 5 h at room temperature. After washing in 0.05% PBS-Tween again, 50 µl of 2-fold dilutions of the standard prepared in 1% PBS-gelatin and 50 µl of samples was added to the plate. The plate was incubated at 4°C overnight for cytokine ELISAs and at

37°C for 1 h for the toxin ELISAs. It was then washed and 50 µl of the secondary antibody diluted in 1% PBS-gelatin was added to the plate. For the toxin ELISAs, after incubation at 37°C for 1 h, the plate was washed and 50 µl of the HRP-conjugated detection antibody diluted 1:1000 in 1% PBS-gelatin was added to all the wells. The plate was incubated at 37°C for 1 h. For the cytokine ELISAs, after incubation at room temperature for 5 h and washing, 50 µl of 1:1000 Streptavidin-peroxidase (KPL) prepared in 1% PBS-gelatin was added to each well to react with the Biotin-conjugated secondary antibody. The plate was incubated at 37°C for 30 min. After washing thoroughly, 100 µl of TMB (3,3',5,5'-tetramethylbenzidine) substrate was added to the wells and the plate was allowed to develop. The reaction was stopped by the addition of 100 µl 0.2 M sulphuric acid to each well. The A_{450} was measured using a plate reader. All the reactions were performed in duplicate. Negative controls with only 1% PBS-gelatin were also maintained in duplicate. The results obtained were analysed using the GraphPad Software Prism 4.0. Nonlinear regression analysis was used to determine the concentrations of toxins in the samples from the standard curves generated.

2.5.5. Preparation of standards

Toxin A was purchased from Calbiochem®, Merck and toxin B was purchased first from Sigma-Aldrich and then from Calbiochem®, Merck. As per the manufacturer's instructions, vials containing the toxins were centrifuged prior to opening to prevent any loss of material. Lyophilised toxin (2 µg) was dissolved in 200 µl DEPC treated water to obtain a concentration of 10 µg/ml. This stock was stored at 4°C. For the cytokine ELISAs, human-recombinant proteins for TNF-α, IL-1β, IL-6, IL-10 and IL-12 (eBioscience), and IL-8 (PeproTech EC) were reconstituted and diluted as per the manufacturer's instructions. These standards were aliquoted and stored at -20°C.

2.5.6. Development of ELISAs

Checkerboard assays were performed to determine suitable antibody concentrations to be used in ELISAs. Recommended dilutions of primary antibody were prepared in 1% PBS-gelatin and 50 µl was coated onto ELISA plates. After blocking in 100 µl

blocking solution, 50 µl of 2-fold dilutions of standards were added to the plates and incubated. Then, 50 µl of recommended dilutions of secondary antibody was added, followed by incubation with 50 µl 1:1000 of detection antibody or Streptavidin-peroxidase, depending on the ELISA. The plates were developed with 100 µl TMB. The reaction was stopped with 100 µl sulphuric acid and A_{450} was measured. Standard curves were generated for the different combinations of antibody concentrations tested. From these, the concentrations of primary and secondary antibodies that generated the best-fit curves over the range of standard concentrations tested were determined. These were used for future experiments.

2.6. Cell culture and related assays

2.6.1. Cell lines

The THP-1 cell line, a human monocytic cell line derived from the peripheral blood of a 1-year old male with acute monocytic leukaemia, was used to measure cytokine production in response to various *C. difficile* antigens. Vero cells, derived from the kidney epithelial cells of the African green monkey, were used for cytotoxicity assays and adherence assays. For adherence assays, additional cell lines were used - Caco-2, human epithelial colorectal adenocarcinoma cells, HT29-16E (HT29-MS), human colonic adenocarcinoma cells and a non-mucus secreting derivative of HT29-16E (HT29-NMS). The cells were recovered from stocks frozen in liquid nitrogen by rapidly thawing at 37°C, adding 15 ml of pre-warmed medium to dilute the cryoprotectant and centrifuging at 1000 g for 5 min. Cells were re-suspended in fresh medium, counted and diluted to a suitable concentration. All cell lines were maintained in a humid incubator at 37°C with 5% CO_2.

2.6.2. Cell counts

Cell suspensions were mixed with equal volumes of Trypan blue solution (Fluka) and allowed to stand for 5 min. Trypan blue was excluded from viable cells which were counted using an improved Neubauer counter:

viable count = no. of cells counted x dilution factor x 10^4

where, no. of cells counted = average of no. of cells in 5 squares

dilution factor = 2

Cells were maintained at a concentration of 0.5-1x10^6 cells/ml.

2.6.3. Culture and passaging of cells

THP-1 cells were initially cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 20% foetal bovine serum (FBS), 6 mM L-glutamine, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) with added Pen-Strep (100 U/ml penicillin, 10 µg/ml streptomycin) to prevent bacterial contamination. FBS was heat inactivated (HI) at 56°C for 35 min with constant shaking and then filter sterilised. This was the initial growth medium (IGM). Cells were passaged up to three times in IGM until they started proliferating rapidly. They were then transferred to the normal growth medium (NGM) comprising of RPMI-1640 medium supplemented with 6 mM L-glutamine and 10% HI-FBS, with Pen-Strep. Cells were maintained in NGM. Frozen stocks were regularly prepared by freezing approximately 10^6 cells/ml in FBS containing 3% glycerol as the cryoprotectant.

Vero cells, Caco-2 cells and both types of HT29-16E cells were cultured in the same way in Dulbecco's minimal eagle medium (DMEM, Sigma-Aldrich) supplemented with 10% HI-FBS, 1% non-essential amino acids and Pen-Strep. The cells were checked regularly by microscopic examination. When the monolayers reached 90% confluence, they were passaged by washing with pre-warmed PBS, followed by treatment with 0.05% trypsin-EDTA (Sigma-Aldrich) at 37°C for 5 min. Warm medium was then added to the cells; FBS in the medium inactivated the trypsin. The typsinized cells were scraped off and collected by centrifugation at 1000 g for 5 min. They were re-suspended at a concentration of 5x10^5 cells/ml in fresh medium. Frozen stocks were regularly prepared by freezing approximately 5x10^5 cells/ml in DMEM supplemented with 20% HI-FBS with 10% DMSO (Sigma-Aldrich) as the cryoprotectant.

2.6.4. Mycoplasma detection

The MycoSensor PCR Assay Kit (Stratagene) was used to detect Mycoplasma infections in the THP-1 cell line when culturing was proving difficult. Cells were cultured in NGM without Pen-Strep for 7 d to maximise the strength of the signal. THP-1 cells were allowed to grow for 48 h after passaging. Then, 100 µl of the culture supernatant was collected by centrifugation at 16000 g for 30 s and diluted 1:10 in DEPC water. It was subsequently boiled at 100°C for 5 min and centrifuged at 16000 g for 5 s. The StrataClean resin was re-suspended by vortexing for 30 s and 10 µl of the resin was added to the supernatant and mixed gently. The sample was centrifuged for approximately 10 s to pellet the resin. The resulting supernatant was removed to a fresh tube and used as the template in the PCR.

A PCR reaction (50 µl) was set up with 10 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$, 200 µM of each dNTP, 2 U Taq and 5 µl of the prepared supernatant. A negative control was maintained with 5 µl DEPC water and a positive reaction was included with 5 µl of the positive control template. An additional reaction was set up which also included 4 µl of the provided internal control. The thermal profile used was:

35 amplification cycles:

Denaturation	94°C	30 s
Annealing	55°C	1 min
Extension	72°C	1 min

The products were visualised on a 1.5% agarose gel as described in 2.3.11.

2.6.5. Differentiation of THP-1 cells and confirmation by flow cytometry

Flow cytometry was used to confirm the differentiation of the monocytic THP-1 cells into macrophage-like cells. THP-1 cells were diluted to $5x10^5$ cells/ml and challenged with different concentrations of PMA (phorbol,12-myristate,13-acetate, Sigma -Aldrich). At 24 h, four 1 ml aliquots of cells challenged with 100, 50, 10 and 5 ng/ml PMA respectively were collected along with four 1 ml aliquots of untreated

cells by centrifugation at 1000 g for 5 min. Cells were washed with 200 µl of buffer (1% PBS-BSA) before staining with fluorescent antibodies for CD4 and CD11b surface markers. From each group, one sample was used as an unstained control for flow cytometry, one sample was stained with fluorescein isothiocyante (FITC) anti-human CD4 antibody (eBioscience), one with allophycocyanin (APC) anti-human CD11b antibody (eBioscience) and one was double-stained with both antibodies. The unstained samples were washed once and re-suspended in 100 µl of buffer. Staining reactions were set up in 100 µl volumes. For CD4 single staining, 5 µl of antibody was added to cells, for CD11b single staining, 20 of µl antibody was used and for double staining, 5 µl of anti-CD4 and 20 µl of anti-CD11b were added to the reactions. Cells were incubated at 4°C for 40 min in the dark. After staining, they were washed twice in 200 µl of buffer and fixed in 10% paraformaldehyde (PFA) at room temperature for 10 min. They were washed again, re-suspended in 100 µl of buffer and stored at 4°C in the dark until they were run on the BD FACSCalibur™ (BD Biosciences) machine. The data were analysed using the FlowJo 9.0 software.

2.6.6. Stimulation of differentiated THP-1 cells with antigens

For the antigen-challenge assays, THP-1 cells were counted and adjusted to a density of $5x10^5$ cells/ml in NGM containing either 10 ng/ml or 50 ng/ml PMA (phorbol,12-myristate,13-acetate, Sigma-Aldrich). To each well of a 96-well tissue culture plate (Greiner), 100 µl cells was added and incubated at 37°C for 24 h for differentiation into macrophage-like adherent cells. After incubation, cells were washed with 100 µl of pre-warmed PBS and serial dilutions of the *C. difficile* antigens prepared in NGM were added to the wells. LPS from *E. coli* R1 (100 ng/ml) was used as a control. Supernatants were collected at 4 h for TNF-α detection and at 24 h for the detection of IL-1β, IL-6, IL-8, IL-10 and IL-12p70. They were stored at -20°C until use.

2.6.7. Cytotoxicity assay

Vero cells (50 µl) at a concentration of $5x10^5$ cells/ml were added to each well of a 96-well tissue culture plate and incubated overnight at 37°C with 5% CO_2 to allow formation of monolayers. Cells were washed in pre-warmed PBS and the medium

was replaced with dilutions of *C. difficile* culture supernatants prepared in DMEM. Samples (50 µl) were added to wells in duplicate. Negative controls were maintained which contained only medium. For the generation of standard curves, *C. difficile* toxin B (Calbiochem®, Merck) was diluted in DMEM to a concentration of 250 ng/ml and 2-fold dilutions were added to the plate in duplicate to a minimum concentration of 0.25 ng/ml. *C. difficile* toxin A (Calbiochem®, Merck) diluted to 125 ng/ml was added to two wells to check for its effect on the assay. Further, to confirm that the observed cytotoxicity was due to *C. difficile* toxins alone, a neutralisation assay was performed with *C. sordellii* antitoxin (Pro-Lab Diagnostics). Samples were prepared with 5 µl supernatant, 5 µl of antitoxin and 40 µl of medium and added to the wells of a different plate in the manner described above. The plates were incubated at 37°C with 5% CO_2 for 48 h.

The plates were checked microscopically and an MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to quantify the cytotoxicity caused by toxin B. The plates were washed twice with pre-warmed PBS and 20 µl of 5 µg/ml thiazolyl blue tetrazolium bromide (Sigma-Aldrich) was added to the wells, followed by incubation at 37°C with 5% CO_2 for 4 h. Formazan produced by cells was dissolved in 100 µl of DMSO and A_{570} was measured. Standard curves generated from the dilutions of toxin B were used to quantify the amounts of toxin B in the culture supernatants.

2.6.8. Adherence assay

A sterile coverslip was added to each well of 6-well tissue culture plates (Greiner) for the adherence assays. Vero cells, Caco-2 cells and both types of HT29-16E cells (1 ml) were added separately to the coverslips in the 6-well plates at a concentration of $2x10^5$ cells/ml and incubated at 37°C with 5% CO_2 to allow formation of monolayers over the coverslips. Except for the Vero cells that were incubated for 48 h, the other cell lines were incubated for 7 d before the assay was performed. *C. difficile* starter cultures were inoculated the day before the assay was to be performed and incubated anaerobically at 37°C overnight.

The starter culture (1 ml) was inoculated into 9 ml of pre-reduced AIM and the medium from the cell lines was replaced with antibiotic-free medium. The bacterial culture was allowed to grow for approximately 4 h until an OD_{600} of 0.5-0.7 was achieved. The medium from the cell lines was replaced with 1 ml of the bacterial suspension and the plates were incubated at 37°C anaerobically for 3 h to allow for attachment of the bacteria to the cell lines. The non-adherent bacteria were washed off the coverslips with sterile PBS and the attached bacterial cells and monolayers were fixed to the coverslips with 100% methanol at 4°C for 10 min. After washing thrice with sterile PBS, the cells were stained with crystal violet for 5 min at room temperature. The coverslips were washed again and dried at room temperature. The coverslips were mounted onto glass slides, cell-side down and visualised under 1000X magnification. The number of *C. difficile* cells per field were enumerated; at least 10 fields were per coverslip were investigated. The adherence of strains was compared to each other.

To identify the effect of flagella and S-layer proteins on bacterial adherence, cell lines were pre-incubated with the proteins diluted in the antibiotic-free medium instead of the medium alone before the bacterial cultures were added to them. The bacteria were enumerated in the same way as above. The effect of the proteins of a strain were tested on the attachment of that strain only.

2.7. Real-time RT-PCR

2.7.1. Development of a real-time RT-PCR assay

2.7.1.1. Bacterial strain and genes

C. difficile strain 630 was used as the reference strain to develop a real-time RT-PCR assay to study gene expression in *C. difficile* genes as it was the only fully sequenced genome available at the time this study was started (Sebaihia *et al.*, 2006). The genes of the PaLoc - *tcdA*, *tcdB*, *tcdC*, *tcdR* and *tcdE* - and *spo0A* were studied. Two housekeeping genes were selected - *tpi* (triose phosphate isomerase) and *rrn* (16S rRNA gene).

2.7.1.2. Growth curves

The growth of *C. difficile* strain 630 was studied in two independent growth curves as described in 2.2.3. At every 4 h from the time of inoculation (0 h) to 24 h, OD_{600} readings were taken. Simultaneously viable counts were also performed. The values obtained from the two methods were plotted against time and the correlation between the two determined, so that in future studies cell numbers/ml could be estimated during growth without performing viable counts. This information was required to collect optimal numbers of vegetative cells for RNA extraction at the different time-points and to standardise the number of cells collected at each time-point.

2.7.1.3. RNA extraction and DNase I treatment

The workspace and equipment to be used for RNA extraction were treated with RNase*Zap*® (Ambion) to obtain an RNase-free environment. RNA was extracted using the RiboPure™-Bacteria kit (Ambion) according to the manufacturer's instructions. From 4 h onwards, a standardised volume of culture was collected in duplicate (50 ml at 4 h to 5 ml at 24 h) by centrifuging at 4000 g for 10 min. At 0 h, 50 ml of the culture was collected. The pellets obtained were centrifuged again at 16000 g for 2 min to remove as much medium from them as possible. The dried cell pellets were snap-frozen in 70% ethanol-ice mixture and stored at -20°C. After 24 h, the samples were stored at -80°C for a maximum of 5 d before use.

Before RNA extraction, the pellets were thawed and mixed thoroughly with 350 µl RNA_{WIZ}™. The mixture was added to 250 µl Zirconia beads, vortexed vigorously in a Mini Beadbeater™ (Biospec Products) for 3 min and centrifuged at 16000 g for 5 min at 4°C. Approximately 250 µl of bacterial lysate was transferred to a fresh tube and treated with 50 µl chloroform, mixed vigorously for 30 s and incubated at room temperature for 10 min. After centrifuging the sample at 16000 g for 5 min at 4°C, the 200 µl upper aqueous phase containing RNA was mixed thoroughly with 100 µl of 100% ethanol and RNA was bound to a silica filter. After three washes with buffers provided in the kit, RNA was eluted in a low ionic strength elution buffer that was pre-warmed to 95°C. A total of 100 µl of RNA was collected. The RNA was

treated with 4 µl DNaseI (Ambion) in 11 µl DNase buffer at 37°C for 1 h, followed by deactivation of the DNase with 20 µl DNase Inactivation Reagent (Ambion) for 2 min at room temperature. The samples were centrifuged at 16000 g for 1 min to pellet the DNase Inactivation Reagent. The RNA was aliquoted into fresh RNase-free tubes and stored at -80°C. Before storage, the samples were run on a 1.5% agarose gel with a 1 kb DNA ladder and visualised under UV light. The quantity and quality of RNA were assessed using an ND-1000 spectrophotometer. The concentration of RNA was determined using the elution solution as the blank and the purity was determined from the ratio of absorbance at 260 nm to that at 280 nm ($A_{260/280}$).

2.7.1.4. Reverse transcription (RT)

Complementary DNA (cDNA) was prepared from the RNA extracted from cells collected at 4, 8, 12, 16, 20 and 24 h using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. In each reaction, 2 µg RNA was added to 4 µl VILO™ Reaction Mix containing buffered random primers, $MgCl_2$ and dNTPs and 2 µl SuperScript® Enzyme Mix containing SuperScript® III RT, RNaseOUT™ Recombinant Ribonuclease Inhibitor and a propriety helper protein and the volume was made up to 20 µl with DEPC water. The mixture was gently vortexed and incubated at 25°C for 10 min followed by incubation at 42°C for 60 min. The reaction was terminated by incubation for 5 min at 85°C. The quantity and quality of the cDNA samples were assessed using an ND-1000 spectrophotometer. A cDNA pool was prepared with 15 µl cDNA from each of the six samples. Further, the individual samples were diluted 40-fold in order to achieve a concentration equivalent to 50 ng RNA. All the cDNA samples were aliquoted and stored at -20°C.

2.7.1.5. Primer designing

Primers for the eight genes were designed using the Primer 3 software (Rozen & Skaletsky, 2000) based on the genome of *C. difficile* strain 630 (Sebaihia *et al.*, 2006). The primers for all the genes in the study were designed such that they yielded products of 100 bp for all the genes to facilitate inter-gene comparison of

transcription as the Ct value obtained during the amplification of the products would not be affected by the product size. The primers were also designed to have similar temperatures of melting (Tm) to enable running reactions of multiple genes simultaneously. The primers and their characteristics are listed in Table 2.3.

Table 2.3. Primers used in this study and their characteristics

Gene	Name	bp	Tm	GC%	Primer sequence
tcdA	A1	20	60.38	50.00	5' GCTATTGCTGCAGTCGGATT 3'
	A2	22	59.02	45.45	3' TACCATTAACAGTCTGCCAACC 5'
tcdB	B1	20	60.54	50.00	5' TGGTGAAGATGGTGTCATGC 3'
	B2	22	59.53	40.91	3' TTCTCCCTCAAAATTCTCATCC 5'
tcdC	C1	23	59.69	39.13	5' TTTAAGAGCACAAAGGGTATTGC 3'
	C2	21	60.24	52.38	3' TGACCTCCTCATGGTCTTCAG 5'
tcdR	R1	24	57.30	33.33	5' AACTCAGTAGATGATTTGCAAGAA 3'
	R2	23	57.20	34.78	3' TTAAATCTGTTTCTCCCTCTTCA 5'
tcdE	E1	26	59.64	34.62	5' AAATATGTGCTTATGTGGATTACCAG 3'
	E2	24	59.49	33.33	3' TTCATCCTTAGCATTCATTTCATC 5'
spo0A	S1	20	56.75	45.00	5' TGTTGAGCTTTTAGGTGCAG 3'
	S2	23	59.90	34.78	3' TGCATGTCTTATTGCTCTTTCAA 5'
tpi	T1	22	59.54	45.45	5' ACTGCTGAAGATGCTAATGACG 3'
	T2	26	60.17	34.62	3' TTCCACCGTATTGTATTCTAACTTCA 5'
rrn	16S1	20	60.02	50.00	5' AGTGAAAGGCTACGGCTCAA 3'
	16S2	20	59.90	50.00	3' CTACGCATTTCACCGCTACA 5'

2.7.1.6. Primer testing

The primers were tested in a conventional PCR with gDNA extracted from strain 630 in 50 µl reactions. Each reaction contained 2 µM of each primer, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 1 U of Taq polymerase, 0.2 mM of each dNTP and 5 µl of gDNA. The thermal profile used for the PCR was as follows:

Initial denaturation	95°C	2 min
40 amplification cycles:		
Denaturation	95°C	1 min
Annealing	56°C	1 min
Extension	72°C	1 min
Final extension	72°C	10 min

The products obtained were checked by electrophoresis as described in 2.3.12. The primers were then tested at different concentrations in a real-time PCR experiment using the Mx3000P QPCR system (Stratagene). Three reactions were set up for each gene containing 2 µM, 1 µM and 500 nM of each primer. Each reaction contained 25 µl SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma), 5 µl gDNA of strain 630, the primers and DEPC water up to 50 µl. The thermal profile used was as follows:

Initial denaturation	95°C	3 min
40 amplification cycles:		
Denaturation	95°C	20 s
Annealing	56°C	20 s
Extension	72°C	20 s
Final dissociation	95°C	1 min
	50°C	1 min
	95°C	30 s

Amplification of the products was checked from the Ct value and the specificity of the primers was determined from the Tm of the products obtained in the dissociation curves generated by the Mx3000P QPCR software (Stratagene).

2.7.1.7. Primer optimisation

Four primer concentrations were selected for preliminary testing for each gene - 50 nM, 100 nM, 200 nM and 400 nM. Real-time PCR reactions were performed as above and negative controls with DEPC water were maintained. From these, two

suitable concentrations were selected for further testing. For *tcdE* alone, two higher concentrations were selected. The selected primer concentrations from these experiments were tested again in duplicate before the final primer concentrations for each gene were selected.

2.7.1.8. Standard curves

Standard curves were performed in duplicate for each gene with the selected primer concentrations in order to determine the efficiency of the individual PCR reactions. Four-fold dilutions of gDNA were prepared such that the amount of gDNA in the samples ranged from 800.0 ng to 12.5 ng.

For each gene, PCR reactions were set up for each gDNA concentration with the selected primer concentrations in 20 µl reactions containing 10 µl SYBR® Green JumpStart™ *Taq* ReadyMix™ using the thermal profile above. The standard curves were obtained from the Mx3000P software. From these, the efficiency of the PCR reactions for each gene was determined. The range of DNA quantity over which the PCR reactions were most efficient was also determined. The standard curves were repeated with 4-fold dilutions of the cDNA pool prepared, such that the reactions contained cDNA from 775.0 ng to 12.0 ng.

2.7.1.9. Real-time PCR

Real-time PCR was performed for the 4, 8, 12, 16, 20 and 24 h samples. The cDNA samples were suitably diluted. Each 20 µl reaction contained 2 µl cDNA, 10 µl SYBR® Green JumpStart™ *Taq* ReadyMix™, the selected primer concentrations and DEPC water. On each plate, duplicate reactions were performed for three genes of interest and the housekeeping gene at the six time-points. Also, duplicate reactions were set up with the dilutions of the cDNA pool to obtain the efficiency of the PCR for each gene in the run. RNA (no RT) controls were included to check for contamination of the RNA preparations with gDNA. DEPC water controls were also included to identify contamination of the PCR reagents and primers, if any. Positive

controls with gDNA were maintained. The PCR was performed using the thermal profile described in 2.7.1.6.

2.7.1.10. Analysis

The data for each run was generated by the Mx3000P software and the Ct values and amplification curves of each sample was checked. Further, the dissociation curves were checked to check the product specificity. The efficiency of the PCR reaction for each gene was determined from the standard curves generated. The expression of the genes of interest at each time-point was normalised to that of *rrn* at that time-point using the Ct value at 4 h as the calibrator or base-line for the expression and was calculated by the Pfaffl method (Pfaffl, 2001) as follows:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target (control-sample)}}}}{(E_{\text{ref}})^{\Delta Ct_{\text{ref (control-sample)}}}}$$

where, target = gene of interest

ref = housekeeping gene

E_{target} = Real-time PCR efficiency of the gene of interest

E_{ref} = Real-time PCR efficiency of the reference gene

$\Delta Ct_{\text{target}}$ = Ct value $_{\text{target}}$ at 4 h - Ct value $_{\text{target}}$ at 8 / 12 /16 / 20 / 24 h

ΔCt_{ref} = Ct value $_{\text{ref}}$ at 4 h - Ct value $_{\text{ref}}$ at 8 / 12 /16 / 20 / 24 h

2.7.2. Real-time PCR

2.7.2.1. Bacterial strains and growth

Growth curves were performed over 24 h for *C. difficile* strains 630 and VPI 10463 and ribotypes 027, 001 and 106 in 300 ml volumes as described in 2.2.3. Growth was assessed by OD_{600} measurement only.

2.7.2.2. RNA extraction

RNA was extracted by three methods: using the RiboPure™-Bacteria kit (Ambion) as described in 2.7.1.3, RNAwiz™ (Ambion) or TRIzol® (Invitrogen) according to

the manufacturer's instructions. At every 4 h from 4 h to 24 h, standardised volumes of culture were collected as described in 2.7.1.2 and 2.7.1.3 in duplicate.

When using RNAwiz™ or using TRIzol®, cell pellets were treated immediately with 500 µl of either reagent, vortexed vigorously in a Mini Beadbeater™(Biospec Products) for 3 min and stored at -80°C for a maximum of 7 d before use. Before RNA extraction, pellets were thawed and incubated at room temperature for 5 min to allow for the dissociation of nucleoprotein complexes. Chloroform (100 µl) was added to the samples and they were mixed thoroughly for approximately 30 s and incubated at room temperature for 10 min. The mixture was subsequently centrifuged at 12000 g for 15 min at 4°C. The upper aqueous phase containing the RNA was transferred to a new tube and RNA was precipitated with 250 µl isopropanol after incubation at room temperature for 10 min and centrifugation at 12000 g for 10 min at 4°C. The supernatant was discarded and the RNA pellet was washed with 500 µl of 75% ethanol followed by centrifugation at 7500 g for 5 min at 4°C. The pellet was air-dried for approximately 10 min and re-suspended in 100 µl DEPC water. DNaseI treatment was performed and the quantity and quality of the RNA were assessed using an ND-1000 spectrophotometer.

2.7.2.3. Reverse transcription

cDNA was prepared using the SuperScript® VILO™ cDNA Synthesis Kit according to the manufacturer's instructions as described in 2.7.1.4. The quantity and quality of the cDNA samples were assessed using an ND-1000 spectrophotometer.

2.7.2.4. cDNA pool and dilutions

cDNA (2 µg) was diluted 4-fold to get a starting concentration equivalent to 500 ng RNA. A cDNA pool was prepared using 15 µl cDNA from each of the six samples. Four-fold dilutions of the 500 ng pool were prepared corresponding to 125, 31.25 and 7.8125 ng RNA. These dilutions were used to generate standard curves. For the genes of interest, individual cDNA samples were diluted 40-fold to achieve a concentration equivalent to 50 ng RNA. For the housekeeping gene, *rrn*, cDNA

samples were diluted to obtain a concentration equivalent to 1 ng RNA. The samples were aliquoted and stored at -20°C.

2.7.2.5. Real-time PCR

PCR reactions were set up in 20 µl volumes with 5 µl of cDNA. On each 96-well plate, reactions were set up in duplicate for three genes of interest and *rrn*. Standard curves for the four genes, negative controls, no-RT controls and positive controls were also run in duplicate. The thermal profile used was as described in 2.7.1.6. The data was analysed as before.

2.8. Sensitivity assays

2.8.1. Antibiotics and agents

Eight antibiotics were selected for antimicrobial susceptibility testing (AST) of the *C. difficile* strains. These were vancomycin, metronidazole, ceftriaxone, tetracycline, erythromycin, clindamycin, ciprofloxacin and moxifloxacin. The concentrations tested and the guidelines for interpretation of the results are listed in Table 2.4.

Table 2.4. Antibiotics used in this study and interpretation guidelines

Antibiotic	Range of concentrations tested (µg/ml)	MIC interpretive criteria (µg/ml)[a]		
		Susceptible	Intermediate	Resistant
Vancomycin (V)	0.5 – 8.0	≤ 2	4 – 16	≥ 32
Metronidazole (M)	0.5 – 8.0	≤ 8	16	≥ 32
Ceftriaxone (C)	16.0 – 256.0	≤ 16	32	≥ 64
Clindamycin (CL)	0.25 – 64.0	≤ 2	4	≥ 8
Tetracycline (T)	0.5 – 64.0	≤ 4	8	≥ 16
Erythromycin (E)	0.25 – 64.0	≤ 2	4	≥ 8
Moxifloxacin (MX)	0.25 – 128.0	≤ 2	4	≥ 8
Ciprofloxacin (CP)	4.0 – 128.0			≥ 16

[a] Adapted from the CLSI criteria for anaerobes and published data (Mutlu *et al.*, 2007).

Five agents were tested for similar studies. These were Actichlor, a disinfectant routinely used in hospitals, Decon 90, a laboratory decontaminant and Microsol 3+, TriGene Advance and Virkon, three commonly-used laboratory disinfectants. The active ingredients of these agents, manufacturers and recommended working concentrations are listed in Table 2.5.

Table 2.5. Agents used in this study

Agent	Active compounds	Manufacturer	Recommended working concentration
Actichlor	Sodium dichloroisocyanurate	Ecolab	1000 ppm chlorine
Decon 90	Anionic and non-anionic surfactants	Decon Laboratories Ltd.	1:10 dilution
Microsol 3+	Tertiary alkylamine and quaternary ammonium compounds	Anachem Ltd.	1:10 dilution
TriGene Advance	Polymeric biguanide hydrochloride and organic quaternary compounds	Medichem International Ltd.	1:100 dilution
Virkon	Potassium peroxymonosulfate	Antec International Ltd.	1:100 dilution

2.8.2. Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations were determined by three methods: Wadsworth agar-dilution method, E-test and broth-microdilution. The agar-dilution method was used to determine the MICs of antibiotics and disinfectants, the E-test was used only for antibiotics and broth-microdilution was used only for disinfectants. For all three methods, the *C. difficile* strains were grown on blood agar at 37°C for 24 h anaerobically. Growth was lifted from five or more colony edges and inoculated into 3 ml of pre-reduced thioglycollate broth (Oxoid) supplemented with 5 µg/ml haemin and 1 µg/ml vitamin K_1 (NCCLS, 2004) and incubated at 37°C anaerobically overnight. The optical density of the cultures was adjusted to a 0.5 McFarland standard by addition of pre-reduced broth and these were used for MIC testing.

For the agar-dilution method, antibiotic stocks were prepared at 10000 µg/ml, which were diluted to a concentration equivalent to ten times (10X) the initial concentration to be tested and disinfectant stocks were prepared at 10X of the manufacturer's recommended working concentration in distilled water. Two-fold dilutions of the stocks were prepared in distilled water till 10X of the lowest concentration to be tested was achieved. The medium used for the test was Brucella agar (Oxoid) supplemented with 5% defibrinated horse blood, 5 µg/ml haemin and 1 µg/ml vitamin K_1. The test plates were prepared by adding 1 ml defribrinated horse blood, 2 ml of the test agent and 17 ml cooled molten agar. The plates were pre-reduced for 2 h and inoculated with a sterile multi-point inoculator and incubated anaerobically. All inoculations were carried out starting from the plate with the lowest concentration of agent. In addition to the test plates, control plates prepared with distilled water were inoculated to check for contamination: two plates were inoculated before the test plates, two were inoculated after them and two were inoculated in between different test agents. Of each of these sets, one plate was incubated aerobically and one anaerobically. Growth on the plates was checked after 24 h and 48 h of incubation at 37°C and the MIC of each agent for each strain was determined as the lowest concentration of the agent to inhibit visible bacterial growth. The E-test was only employed for moxifloxacin which was unavailable in powdered form. Standardised cultures were swabbed onto pre-reduced enriched Brucella agar plates prepared as the control plates in the agar-dilution method, such that a lawn of growth would be obtained. Test strips, containing an exponential gradient of antibiotic, were placed smoothly on the surface of each plate. The plates were incubated anaerobically at 37°C and checked at 24 and 48 h for growth. The MIC was determined as the concentration of antibiotic indicated on the strip where the zone of inhibition began. The tests were performed in triplicate.

MICs by broth-microdilution were performed in 96-well plates (Greiner). Two-fold dilutions of test agents were prepared in pre-reduced AIM and 100 µl of each dilution was added to the plate in duplicate. Standardised culture (10 µl) was added to each well. Positive controls (medium and culture) and negative controls (medium and

agent) were maintained. Negative controls were required for each agent to quantify turbidity resulting from it being dissolved in AIM rather than water. The plates were incubated anaerobically at 37°C for 48 h. OD_{600} was measured after shaking for 5 min. The lowest concentration of agent to inhibit visible bacterial growth was recorded as the MIC. The experiments were performed in triplicate.

2.8.3. Preparation of spores

C. difficile cultures (500 ml) were incubated anaerobically at 37°C for 7 d. The cultures were collected by centrifugation at 4000 g for 10 min. Alcohol-resistant spores were prepared as described in 2.2.5. The number of spores in each sample was determined from serial dilutions. The spore preparations were standardised to a concentration of 10^6 spores/ml by dilution in distilled water.

2.8.4. Spore viability assays

The agents were tested for their activity against spores in a suspension test at the recommended working concentration and 1/2 and 1/5 of the same. For each test, 100 µl of spore suspension was added to 900 µl of each dilution and mixed thoroughly. At 2, 10 and 30 min, 100 µl of the test was inoculated into 900 µl pre-reduced AIM, mixed and incubated at 37°C for 5 d in duplicate. Positive controls without agent and negative controls without spores were maintained. The tubes were examined for growth. Samples from tubes with no visible growth were plated to identify cidal or static activity. These were examined for growth after 5 d of anaerobic incubation.

2.8.5. Determination of log_{10} reduction

To determine log_{10} reduction in spores in suspension tests, spores were treated with agents at the recommended working concentration in the presence or absence of organic matter. For tests without organic matter, 100 µl of spores (approximately 10^4 spores) in distilled water was added to 900 µl of disinfectant. After 10 min, the spores were collected by centrifugation at 16000 g for 2 min. The pellets were washed twice with distilled water. The spores were re-suspended in 1 ml distilled water and 100 µl of this suspension was plated in duplicate and incubated at 37°C

anaerobically for 48 h. Positive controls were maintained. For tests in the presence of organic matter, 0.27% BSA was introduced. The reduction was calculated as:

$\log_{10}$ reduction = $\log_{10}(N_0/N_{10})$,

where, N_0 = the number of spores in the positive control, and

N_{10} = the number of viable spores recovered from the test at 10 min.

2.8.6. Surface decontamination testing

Five hard, non-porous surfaces were used in this study - aluminium, glass, plastic, vinyl self-adhesive tile and white ceramic tiles. Surfaces were autoclaved and dried in a hot-air oven before use; the vinyl tiles could not be autoclaved and were cleaned with 70% ethanol for 10 min before use. Once prepared, squares of approximately 1 cm^2 were marked out on each surface with a wax crayon. The squares were artificially contaminated with 10 µl of spore suspension (approximately 10^3 spores) of one *C. difficile* strain and left to air-dry for 2 h. Subsequently, 50 µl of cleaning agent at the recommended working concentration was added to the dried spore suspension. At 2 min and 10 min, the area was scratched with a pipette tip 10 times to mimic scrubbing. The agent was aspirated from the surface and the area was washed with 100 µl distilled water. The aspirated agent and wash were added to 850 µl distilled water to obtain a final volume of 1 ml. An aliquot (100 µl) of this was plated on blood agar and incubated anaerobically for 48 h. Positive controls were maintained, in which only water was used for cleaning. Negative controls were also included using uncontaminated squares on each surface. The $\log_{10}$ reduction was calculated (2.8.5) from two independent experiments.

2.8.7. Effect of sub-MIC concentrations on sporulation

C. difficile strains were cultured in media containing 1/4 MIC of each agent as determined from 2.8.2 to study the effect of sub-MIC concentrations on growth and sporulation. The agents were diluted in AIM with and without 0.5% sodium taurocholate. The dilutions (200 µl) were added to the wells of a 24-well plate (Greiner) and allowed to pre-reduce for 4 h. Starter cultures were prepared (2.2.2)

and 1 ml of this culture was added to 11 ml of pre-reduced AIM and incubated anaerobically at 37°C for approximately 4 h until an OD_{600} of 0.3 (±0.05) was achieved. Positive controls without agents and negative controls without culture were maintained. The experiments were performed in duplicate. Following incubation, OD_{600} of the cultures was measured to check the growth of the cultures. They were left under aerobic conditions for 24 h to ensure death of the vegetative cells. Spore production was determined by plating 10-fold serial dilutions of the cultures on blood agar and incubating anaerobically for 5 d.

2.9. Detection of environmental contamination

2.9.1. Sampling

To examine the prevalence of environmental contamination with *C. difficile*, 93 sites in and around our laboratory were sampled with contact plates. Sampling was performed in duplicate; for 40 sites, one CCEY agar and one blood agar plate were used and for the remaining 53 sites, two blood agar plates were used. The plates were incubated at 37°C for 5 d. The total load of anaerobic bacteria and the percentage of *C. difficile* were calculated from the colonies obtained on the plates.

2.9.2. DNA extraction and typing

The suspected *C. difficile* isolates were sub-cultured on blood agar for 48 h and their identity was confirmed by colony morphology, odour and the characteristic golden-yellow fluorescence under UV light. DNA extraction was performed using Chelex®100 (BioRad) (2.3.1) followed by ribotyping (2.3.2).

2.10. Statistical analysis

All statistical tests were performed using the GraphPad Software Prism 4.0. Non-linear regression analysis was performed for assays involving standard curves. Inter-strain comparisons were performed by area under curve (AUC) analyses and 1-way analysis of variance (ANOVA) testing.

3. Growth-related virulence of *C. difficile*

3.1. Introduction

Virulence is a measure of the degree to which a pathogen can cause disease in a host. For bacteria, the conditions and rate of growth are closely related to the production of virulence factors. The virulence of *C. difficile* is primarily dependent on the production of its spores and its two toxins - toxin A and toxin B - which together maintain the cycle of *C. difficile* infection and transmission. This study investigates whether the level of expression of toxins and spores varies between *C. difficile* strains was investigated.

3.1.1. Role of growth rate in virulence

The growth rate of a microorganism is an important factor in its pathogenesis and can contribute to the outcome of an infection (Brown & Williams, 1985; Smith, 1990). In some organisms, it has been observed that virulence increases with the growth rate (Marsh *et al.*, 1994). A fast growing organism can overcome the initial non-specific immune response generated by the host, allowing the disease to be established before the specific response can be generated (Smith, 1990). Rapid growth of organisms can be beneficial if receptors for adhesins are expressed earlier and adherence to cells can be induced sooner. It can also compensate for the loss of bacterial numbers at the primary site of infection. In acute disease, rapid growth is required to evade the initial non-specific immune response. In chronic infection, however, the slower the growth rate, the less the immune response generated and the longer the pathogen can persist within the host and cause disease. For a carrier status, an extended resistant stationary phase may be beneficial to the organism. Thus, the growth rate is significant in the type of infection established and the outcome of disease. Growth rates is governed by the internal environment of the host and the site of infection. The size of the pathogen population depends not only on the growth rate, but also on the tissue's ability to sustain it (Smith, 2000).

The growth of organisms *in vivo* can vary significantly from growth *in vitro* as it is dependent on the host environment (Smith, 1990). This observation calls into questions the use of in-vitro studies altogether. Furthermore, certain virulence factors may only be expressed within the host, but others such as production of siderophores, responses to nutrient limitation and sensitivity to antibiotics can be observed *in vitro* (Brown & Williams, 1985; Gilbert *et al.*, 1990). Thus, in-vitro studies can be used as a starting point for investigation of bacterial virulence factors.

In *C. difficile* infection, the clinical spectrum ranges from mild diarrhoea to fulminant colitis, suggesting that several strains of varying virulence exist (Knoop *et al.*, 1993). However, comparisons between growth of different *C. difficile* strains with varied virulence, including potentially 'hypervirulent' strains, have not revealed any significant variations in growth rates and patterns (Merrigan *et al.*, 2010; Warny *et al.*, 2005) suggesting that growth rate does not influence virulence, at least *in vitro*.

3.1.2. Toxins and spores as virulence factors

The toxins and spores of *C. difficile* are its main virulence factors. During CDI, vegetative cells produce both toxins and spores. The toxins elicit their enterotoxic and cytotoxic effects within the gastrointestinal tract of the host (1.4.1.2) and the damage leads to diarrhoea and disease (1.2.3). During this process, vegetative cells produce spores, which are released into the environment. The spores survive and persist in the environment and are transmitted to susceptible individuals within whom they can germinate into vegetative cells and resume the infection cycle (1.4.3). Sporulation and toxin production were suggested to be alternative mechanisms for survival (Akerlund *et al.*, 2006) but more recently it has been shown that both processes are co-regulated (Underwood *et al.*, 2009).

3.1.2.1. Toxin A or toxin B?

The lethality of *C. difficile* toxins has been a subject of debate since their discovery. Which is more important in disease: toxin A or toxin B? Toxin A was originally observed to cause more severe disease and increase mortality in mice and hamsters

(Taylor *et al.*, 1981) but in another study, toxin B was found to be more lethal to mice per milligram of protein as compared to toxin A (Sullivan *et al.*, 1982). It was then demonstrated that a crude filtrate of *C. difficile* VPI 10463 administered intragastrically to hamsters could cause hemorrhagic fluid to accumulate in the stomach, small intestine and caecum, but when toxin A was removed by immunoadsorption, no diarrhoea or pathology was observed (Lyerly *et al.*, 1985). When only toxin B was administered to the hamsters, no damage was caused even at high amounts, but when a small amount of toxin A was added to toxin B, CDI pathology was observed. Also, toxin B administered to animals with bruised ceca resulted in illness and death. Interestingly, multiple small doses of toxin A which were individually harmless seemed to have a cumulative effect over time. These observations indicated that toxin A was more potent than toxin B and that initial damage by toxin A or physical damage to the intestinal epithelium was required to mediate toxin B cytotoxicity. In another study, it was shown that the intensity of inflammation induced in CDI was not dependent on the concentration of toxin B (Vernet *et al.*, 1989). However, a study showing that toxin B was ten times more potent at inducing morphological damage in human colonic cells than toxin A suggested that it could play an equally important role in the pathogenesis of *C. difficile* (Riegler *et al.*, 1995).

This debate was further complicated by the discovery of A-B+ *C. difficile* strains (Depitre *et al.*, 1993; Kato *et al.*, 1999). The cytopathic effects, substrate specificity and receptor-binding domain of toxin B of these strains were similar to the lethal toxin of *C. sordellii*, possibly owing to recombinational exchange (von Eichel-Streiber *et al.*, 1995). Axenic mice treated with these strains did not develop diarrhoea or die but the animals were successfully colonised and even protected from subsequent infection (Depitre *et al.*, 1993). However, human infection was observed with these strains reaffirming the importance of toxin B in CDI (Drudy *et al.*, 2007a).

A recent study using toxin A and B mutants also showed that toxin B was essential in CDI (Lyras *et al.*, 2009). Toxin A mutants produced wild-type levels of toxin B and

were as lethal to hamsters as wild-type strains, while toxin B mutants were able to colonise hamsters but were lethal to only 21% of the infected animals. The most recent investigation into the importance of the two toxins demonstrated that both toxins were equally essential for virulence (Kuehne *et al.*, 2010). Individual toxin A and toxin B knockout strains were able to cause disease in hamsters, whereas a double-mutant was only able to colonise the animals and not induce any symptoms.

3.1.2.2. Kinetics of toxin and spore release during growth

Growth-related toxin and spore production has been studied in several *C. difficile* strains over the years. In most studies, an exponential phase of 12 hours followed by a stationary phase of 12 hours has been observed (Ketley *et al.*, 1984; Rolfe & Finegold, 1979). Growth was not found to be variable between *C. difficile* strains despite their toxigenic potential (Vernet *et al.*, 1989). Amounts of toxin in culture filtrates were observed to increase in the stationary phase of growth as numbers of vegetative cells decreased (Ketley *et al.*, 1984; Onderdonk *et al.*, 1979; Rolfe & Finegold, 1979). Strains producing greater amounts of toxin A also produced greater amounts of toxin B (Tucker *et al.*, 1990; Vernet *et al.*, 1989). A correlation between the strain of *C. difficile* and the amount of toxin produced was observed. Strains belonging to serogroups A, C, G and H produced lower levels of toxin as compared to VPI 10463 (Karlsson *et al.*, 2003; Wren *et al.*, 1987). Spores were detected only after the exponential phase (Kamiya *et al.*, 1992; Ketley *et al.*, 1986; Onderdonk *et al.*, 1979). These studies together gave a general idea of the behaviour of *C. difficile in vitro* and demonstrated that toxin and spore production in *C. difficile* could vary with the phase of growth and the strain in question. Any variations observed were probably due to differences in the media used or the size of the initial inoculum (Dupuy & Sonenshein, 1998; Hundsberger *et al.*, 1997; Mathis *et al.*, 1999).

3.1.3. Pathogenicity locus

The pathogenicity locus (PaLoc) or toxigenic element of *C. difficile* is a 19.6 kb chromosomal region that codes for toxin A and toxin B (Fig. 3.1) (Hammond & Johnson, 1995). This highly stable and conserved region consists of five open

reading frames (ORFs) - *tcdR* is the first (5') gene in the PaLoc, followed by *tcdB*, *tcdE*, *tcdA* and *tcdC*, respectively (Cohen *et al.*, 2000; Hammond & Johnson, 1995). The PaLoc has features of a distinct genetic element. It has a single integration site, unidirectional orientation, gene with sequences dissimilar to any others on the chromosome and conserved bordering sequences (Braun *et al.*, 1996). It is not an independent mobile genetic element as no transposon-, phage- or plasmid-like elements or inverted repeats characteristic of mobile genetic elements are found adjacent to it (Braun *et al.*, 1996). In non-toxigenic strains, the PaLoc is replaced by a unique conserved 115 bp fragment (Braun *et al.*, 1996; Hammond & Johnson, 1995). This fragment does not contain an ORF but does have several internal repeat regions which are characteristic of an insertion sequence (Hammond & Johnson, 1995). Also, a hairpin loop upstream of the 115 bp fragment which forms a transcription terminator might play a role in the integration and possible recombination of the PaLoc (Braun *et al.*, 1996).

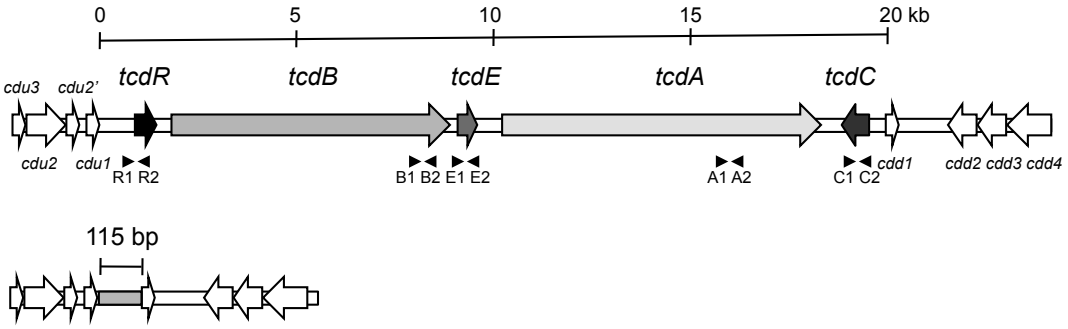
It was originally believed that the PaLoc was necessary for virulence and that only strains producing both toxins could produce clinically significant disease, but disease caused by A-B+ strains has been widely reported (Drudy *et al.*, 2007a; Limaye *et al.*, 2000). Thus, the complete PaLoc is not essential for disease (Cohen *et al.*, 2000). Cases of diarrhoea caused by non-toxigenic *C. difficile* strains have been also reported suggesting that there are other virulence determinants present in *C. difficile* (Martirosian *et al.*, 2005).

3.1.3.1. *tcdA* and *tcdB*

The toxin genes, *tcdA* and *tcdB*, lie within 1.4 kb of each other on the PaLoc and are transcribed in the same direction (Hammond & Johnson, 1995; Hammond *et al.*, 1997). *tcdA* and *tcdB* are genes of 8133 bp and 7089 bp, respectively (Barroso *et al.*, 1990; Dove *et al.*, 1990). Both toxin genes have a G+C content of less than 28% and exhibit a high level of similarity of approximately 66% (von Eichel-Streiber *et al.*, 1990; Voth & Ballard, 2005). Characteristic of clostridial toxin genes *tcdA* and *tcdB* have a very long spacing between the start codon ATG and the transcription start in

the promoter and have stem-loop transcriptional terminators at their 3' ends (von Eichel-Streiber *et al.*, 1992). The genes are 49% identical and 14% of the regions show conserved substitutions (von Eichel-Streiber *et al.*, 1992). The most homologous regions of the toxins are the N- and C-termini. The N-termini show 64% homology, which explains the similarity in their biological activity on the same cellular substrates. This structural and functional similarity suggests that the genes arose by duplication. The C-terminal CROP clusters in the two genes are also highly homologous but those in *tcdB* are more varied. There is only one region in the gene products that has an amino acid homology of only 30% - residues 1441 to 1689 in TcdA and residues 1440 to 1692 in TcdB. The variations in this region and in the C-terminal CROPS might explain the difference in the effects of the toxins *in vivo* and could be the reason why they induce distinctive and specific polyclonal antibodies.

Fig. 3.1. PaLoc of *C. difficile* and ORFs outside it



The pathogenicity locus of *C. difficile* is shown using that of strain VPI 10463 as an example. The 19.6 kb PaLoc contains five open reading frames: the toxin genes *tcdA* and *tcdB*, the positive regulator of toxin production *tcdR*, the negative regulator *tcdC* and *tcdE*, which codes for a holin-like protein involved in the release of toxins. There are four conserved genes upstream of the PaLoc - *cdu3*, *cdu2*, *cdu2'* and *cdu1* - and four conserved genes downstream of it - *cdd1*, *cdd2*, *cdd3* and *cdd4* - which could play a role in regulation or integration of the PaLoc genes. The direction of the arrows indicates the direction of transcription of the genes. The positions of the primers designed for real-time RT-PCR in this study are indicated below the PaLoc map. In non-toxigenic *C. difficile* strains the PaLoc is replaced by a 115 bp DNA fragment which bears similarity to an insertion sequence. Adapted from Braun *et al.*, 1996.

3.1.3.2. *tcdR*

tcdR is the first gene of the PaLoc and is the positive regulator of toxin production. It is a 552 bp gene that lies upstream of *tcdB* and is transcribed in the same direction as the toxin genes (Hammond & Johnson, 1995). The role of *tcdR* in toxin production was first demonstrated in an expression vector system (Moncrief *et al.*, 1997). It was observed that with *tcdR* in *trans*, toxin A and toxin B production was 500-fold and 800-fold higher, respectively, than expression in the absence of *tcdR*. This proved that the toxin genes had their own promoters which could be stimulated by TcdR.

TcdR is an alternate sigma factor (Mani & Dupuy, 2001). It is a basic protein of 184 amino acids and molecular weight of 22 kDa with a C-terminal helix-turn-helix motif characteristic of DNA-binding proteins (Braun *et al.*, 1996; Hammond & Johnson, 1995; Hundsberger *et al.*, 1997). Sequence comparison showed that TcdR was 28% identical to UviA, the positive regulator of bacteriocin production in *C. perfringens* and 21% identical to Orf-22 of *C. botulinum* 468, the positive regulator of neurotoxin C1 production (Hundsberger *et al.*, 1997; Moncrief *et al.*, 1997). These three genes have similar promoters and regulate transcriptional control over their respective toxin genes (Hundsberger *et al.*, 1997; Marvaud *et al.*, 2000).

TcdR regulates the transcription of the PaLoc by mediating binding of the RNA polymerase holoenzyme to promoters of the toxin genes (Mani & Dupuy, 2001). It cannot bind to the promoters itself, but can bind to RNA polymerase in the absence of the promoters and possibly alters the structure of RNA polymerase to stabilise promoter-binding. The promoter of *tcdR* is highly homologous to those of *tcdA* and *tcdB* (Braun *et al.*, 1996). Thus, it is not surprising that along with activating the transcription of the toxin genes, it also activates its own expression and is auto-regulated (Mani & Dupuy, 2001; Mani *et al.*, 2002). *tcdR* regulation is controlled by environmental stimuli through its two promoters: one is involved in basal expression of the gene during the exponential phase of growth and does not require TcdR, while the other is TcdR-dependent and is affected by growth phase and also by the presence of carbon sources such as glucose (Mani *et al.*, 2002).

3.1.3.3. *tcdC*

tcdC is the last gene of the PaLoc and is the negative regulator of toxin production. It is a 695 bp gene located downstream of *tcdA* and is transcribed in the opposite direction to the toxin genes (Dove *et al.*, 1990; Hammond & Johnson, 1995; Phelps *et al.*, 1991; von Eichel-Streiber *et al.*, 1992). It was originally thought that *tcdC* was not required for toxin production (Phelps *et al.*, 1991). However, transcriptional and translational studies showing that its expression was inversely correlated to that of the other PaLoc genes suggested that *tcdC* negatively regulated toxin production (Govind *et al.*, 2006; Hundsberger *et al.*, 1997).

TcdC is a highly acidic 26 kDa protein containing stretches of repetitive amino acids and shows no homology to any known protein (Braun *et al.*, 1996; Hundsberger *et al.*, 1997). It is a membrane-associated protein that can form dimers (Govind *et al.*, 2006; Matamouros *et al.*, 2007). It has a transmembrane domain at the N-terminal and an active domain at the C-terminal (Matamouros *et al.*, 2007). The N-terminal is antigenic but not required for activity. The C-terminal is highly similar to that of *Bacillus cereus* BD-0954 and *Lactobacillus casei* A2 bacteriophage A2-p22 protein and appears to be a conserved domain. The latter proteins are coded near or in temperate bacteriophage genomes suggesting that the PaLoc could have similar origins. Although the regulation of gene expression by transcription factors localised within cell membranes seems unlikely, membrane-associated transcriptional factors have been identified in other organisms such as the RpoE sigma factor of *E. coli* (Dartigalongue *et al.*, 2001).

TcdC specifically represses transcription of the PaLoc by destabilising the TcdR-containing holoenzyme which initiates transcription of the toxin genes (Dupuy *et al.*, 2008; Matamouros *et al.*, 2007). Like TcdR, TcdC cannot directly bind to the promoters of the toxin genes but it can bind to the TcdR-RNA polymerase complex. The TcdR-holoenzyme is more sensitive to TcdC than the TcdR-holoenzyme-promoter complex. TcdC sequesters TcdR in the manner of a classical anti-sigma factor. However, it can also interact directly with RNA polymerase and perhaps

represses toxin production by more than one mechanism. TcdC expression appears to be growth phase-dependent with greater amounts of protein being detected in the membrane fraction of cells in the exponential phase than in the stationary phase of growth (Govind *et al.*, 2006). This is in agreement with expression of *tcdC* seen previously (Hundsberger *et al.*, 1997). However, recent studies have not consistently observed such expression patterns and have suggested that TcdC might have a regulatory rather than a repressive role in toxin production (Merrigan *et al.*, 2010).

The *tcdC* gene has been shown to carry a number of deletions and extensive molecular analyses have been performed to define these mutations. Initially, three *tcdC* genotypes were identified: A, B and C (Spigaglia & Mastrantonio, 2002). *tcdC-A* shows a 39 bp deletion, while *tcdC-B* and *tcdC-C* show an 18 bp deletion. The truncation of TcdC to 61 amino acids from 232 amino acids is observed in *tcdC-A*, while *tcdC-B* and *C* code for the same protein of 226 amino acids. Apart from *tcdC-A* and *tcdC-B*, 15 other unique *tcdC* genotypes have been identified, with 18 bp or 36 bp deletions (Curry *et al.*, 2007). Of these, the *tcdC-sc1* genotype carries an 18 bp deletion along with a single nucleotide deletion at position 117 (Δ117) which introduces a stop codon and truncates the TcdC protein to 65 amino acid residues (Curry *et al.*, 2007; MacCannell *et al.*, 2006). This genotype was found to be conserved in strains belonging to ribotype 027 which produced high levels of toxin (Curry *et al.*, 2007). The 18 bp deletions were initially believed to correlate with greater virulence but this was later found to be untrue (Matamouros *et al.*, 2007). The Δ117 deletion might be of greater significance in virulence (Curry *et al.*, 2007).

3.1.3.4. *tcdE*

tcdE is a 501 bp gene located between *tcdA* and *tcdB* and is believed to have a role in the secretion of toxins from within *C. difficile* cells as the toxins do not have signal peptides for release (von Eichel-Streiber & Sauerborn, 1990).

TcdE is a highly hydrophobic protein of 166 amino acids and molecular weight of 19 kDa (Dove *et al.*, 1990; Hundsberger *et al.*, 1997). It has structural features similar to those of bacteriophage holin proteins such as homologue *yqxH* of *B. subtilis*, holins

of *Streptococcus thermophilus* bacteriophage Sfi19 and of *S. pneumoniae* phage Cp-1 (Tan *et al.*, 2001). The greatest similarity between these proteins was found in the 46 amino acids present at the C-terminal.

The role of *tcdE* was investigated using an expression vector system (Tan *et al.*, 2001). Cells expressing TcdE stopped growing on the induction of *tcdE*; the loss of cell membrane integrity, which was characterised by the fusion of the periplasm and the cytosol and the lack of membrane secretory vesicles, resulted in cell lysis. Thus, TcdE could function as a lytic protein and aid toxin release. However, recent studies have found that TcdE does not induce lysis or affect membrane integrity (Govind & Dupuy, 2010). Instead, it facilitates toxin release by a phage-like system. TcdE, which is predicted to contain three transmembrane domains, could perhaps form gated channels in the membrane specific for the toxins, which would explain the lack of cytoplasmic leakage observed in these studies.

3.1.3.5. Upstream and downstream of the PaLoc

Upstream of *tcdR* lie *cdu3*, *cdu2*, *cdu2'* and *cdu1* which are genes of 238, 939, 192 and 378 bp, respectively (Braun *et al.*, 1996). They are all transcribed in the same direction as the toxin genes. Cdu1 has features similar to those of repressor proteins and DNA-binding proteins and could be an effector molecule in regulation, while Cdu2 and Cdu2' together could function as a Na^+-transporter.

Downstream of *tcdC* are *cdd1*, *cdd2*, *cdd3*, *cdd4* which are genes of 243, 735, 759 and 903 bp, respectively. *cdd1* shares the same orientation as *tcdC*, while the other genes are transcribed in the opposite direction. Cdd2-4 might be functionally-linked proteins that could form an ABC transporter system commonly associated with macrolide resistance in Gram-positive bacteria.

3.1.4. Expression of the PaLoc

3.1.4.1. Transcriptional analysis

Studies by Moncrief and colleagues were the first to suggest transcriptional regulation of toxin genes when they showed that *tcdR* in trans was required for toxin

production and that the mRNAs for the toxin genes were detected in a growth phase-dependent manner (Moncrief *et al.*, 1997). *tcdB* levels began to rise at 8 hours and continued to rise during the stationary phase, while *tcdA* expression did not occur till the late exponential to early stationary phase.

Transcription of the entire PaLoc was first investigated by Hammond and co-workers using hybridisation probes (Hammond *et al.*, 1997). They found that probes designed for *tcdA*, *tcdB*, *tcdR* and *tcdE* all hybridised to a transcript of 17.5 kb but the *tcdC* probe did not. The *tcdA* probe also hybridised to a band of 8.4 kb, while the *tcdB* probe hybridised to two additional bands - one of 8.1 kb and 7.4 kb. Probes for *tcdR* and *tcdE* also hybridised to the 8.1 kb band. From these observations, it was evident that the toxins were being transcribed monocistronically and polycistronically. It was concluded that the toxin A transcript was processed immediately form the 17.5 kb band, while the toxin B transcript was processed from the three different bands. The *tcdR* and *tcdE* probes hybridised with bands of 700 bp and 1 kb, respectively along with the 8.1 kb band and were thus co-transcribed with *tcdB*. Transcription from the full-length transcript and the individual transcripts occurred concurrently suggesting that expression of the toxin genes was co-regulated. Also, the transcription initiator site for the 17.5 kb transcript was identified approximately 236 bp upstream of *tcdR* and for *tcdA* and *tcdB*, the initiator sites were 233 bp and 164 bp upstream of the respective genes indicating regulation at the transcriptional level. Further, very few variations were observed in the intergenic regions of the PaLoc between strains with varying toxigenic potential suggesting that the observed regulation was not a result of differences in the DNA sequences of the regulatory regions.

Hundsberger and colleagues demonstrated growth-related transcription of the PaLoc (Hundsberger *et al.*, 1997). They showed that all five genes were transcribed throughout growth. However, the amount of *tcdC* mRNA produced was high in the exponential phase and decreased in the stationary phase, whereas the opposite transcription pattern was observed for the four remaining genes; transcription of *tcdR*, *tcdB*, *tcdE* and *tcdA* was detected only at basal levels during the exponential

phase and increased during the stationary phase. No read-through transcripts from outside the PaLoc were detected and therefore, transcription was driven from the promoters for *tcdR* and *tcdC*. Two transcription units were observed due to the presence of a bidirectional terminator between *tcdA* and *tcdC* and five transcription initiator sites *ptcdR*, *ptcdB1*, *ptcdB2*, *ptcdE* and *ptcdA* were suggested.

Dupuy and co-workers found that the *tcdB* transcript was initiated within or upstream of *tcdR* and the *tcdA* transcript was initiated upstream of *tcdE*; no second promoter site for *tcdB* was found (Dupuy & Sonenshein, 1998). When *C. difficile* was grown in a medium devoid of glucose, low levels of *tcdA* and *tcdB* mRNA were detected during the early exponential phase but there was a rapid accumulation of mRNAs towards the end of the exponential phase and maximum detection in the early stationary phase. The level of *tcdA* mRNA detected was almost 2-fold higher than that of *tcdB* but the increase in their mRNA and protein levels occurred simultaneously. The correlation between the time of transcription and translation of the toxins suggested that they were co-regulated.

Even in *C. difficile* strain 8864, which has an insertion between *tcdE* and *tcdA* and deletion of the 3' regions of *tcdA* and *tcdC*, all the PaLoc genes were transcribed during growth and the transcription was phase-dependent (Soehn *et al.*, 1998). Expression of *tcdR*, *tcdB* and *tcdE* was low in the early exponential phase but increased later and reached the maximum level in the stationary phase but that of *tcdC* and *tcdA* was independent of growth and remained at a constant low level.

In general, greater levels of toxin mRNA were detected in more toxic strains and more transcripts of *tcdA* than *tcdB* were detected, normally at a ratio of 3:1 (Hammond *et al.*, 1997; Mathis *et al.*, 1999). Although the pattern of transcription of the PaLoc was the same in all the above-mentioned studies, the precise timing of the exponential and stationary phases varied. These differences in times of growth and expression possibly arose owing to factors such as inoculum size and type of medium used (Dupuy & Sonenshein, 1998; Hundsberger *et al.*, 1997; Mathis *et al.*, 1999).

Expression of the PaLoc has been observed to be repressed by the *codY* gene (Dineen *et al.*, 2007). CodY is able to bind strongly to the promoter of *tcdR* and weakly to the promoters of *tcdA* and *tcdB*. It also binds to three sites upstream of *tcdR*. Interestingly, CodY also inhibits the transcription of *tcdC* but to a lesser degree than the other genes. Thus, toxin repression by CodY appears to occur directly through the control of *tcdR* expression.

3.1.4.2. Response to environmental stimuli

Toxin production in *C. difficile* varies in response to the environmental conditions such as available nutrients and changes in temperature. Dupuy and Sonenshein found that toxin mRNAs began to accumulate in the late exponential phase only in glucose-deficient complex media (Dupuy & Sonenshein, 1998). The level of transcription declined significantly in the presence of glucose in a dose-dependent manner; at a concentration of 1% glucose in the medium, toxin production was almost completely repressed. The same effect was observed with fusion vectors expressing *tcdR* and *tcdA* suggesting that repression occurred through the promoters of these genes. Fructose and mannitol also repressed toxin gene expression but this was not seen with non-fermentable sucrose or starch. Buffering the medium to pH 7 had no effect on repression. For *C. difficile* cells grown in brain-heart infusion broth containing glucose, the level of *tcdA* mRNA was 15% lower than that seen in the glucose-deficient tryptone yeast extract medium. The differences in toxin levels correlated with the differences in gene expression. Minimal differences in promoters of the toxin genes suggested the involvement of regulatory mechanisms such as catabolite repression (Dupuy & Sonenshein, 1998).

To further investigate this nutritional link with toxin production, Karlsson and co-workers studied growth and toxin production of *C. difficile* in defined and complex media with and without glucose (Karlsson *et al.*, 1999). In both media, growth rate and growth phase did not affect toxin production. In the defined medium, lower glucose concentrations resulted in lower toxin production but in the complex medium, the opposite effect was observed. Thus, glucose was not exerting catabolite

repression on the toxin genes. When select amino acids were added to the complex glucose-deficient medium, toxin production was notably reduced. This suggested that high toxin production observed in *C. difficile* strains after the exponential phase of growth could be a result of amino acid limitation. The addition of the amino acid mixture to the glucose-containing complex medium had no effect on toxin production. Thus, high toxin production was a result of stress; addition of glucose and removal of biotin led to the concomitant down-regulation of the toxins and other proteins.

The addition of biotin and amino acids such as lysine, glutamine, glutamic acid, asparagine and cysteine have also been shown to inhibit toxin production (Karlsson *et al.*, 2008; Yamakawa *et al.*, 1996; Yamakawa *et al.*, 1998). Addition of cysteine to the growth medium blocked synthesis of the toxins and approximately 40 other proteins which were involved in metabolic pathways utilising alternate energy sources such as succinate, butyrate, carbon monoxide and folate. Further, simultaneous transcription of *tcdA*, *tcdR*, *folD* and *sigH* suggested a common regulatory mechanism and an association between toxin production, alternate energy metabolism and initial sporulation events.

The repression of toxin production by CodY also changed with environmental conditions; greater repression was observed in the presence of branched-chain amino acids and GTP (Dineen *et al.*, 2007). This suggested that toxins were expressed and released only when cells were deprived of nutrients (Dineen *et al.*, 2010).

Toxin production was also found to be temperature-dependent (Karlsson *et al.*, 2003). This dependence was unaffected by the nutritional constituents of the medium in which *C. difficile* was grown. Maximum toxin production was observed at 37°C, with much lower levels of toxin detected at 22°C and 42°C. Corresponding increases in mRNA levels of *tcdA* and *tcdB* were also detected at 37°C. This was a clear indication of adaptation to host conditions. *tcdR* expression was also temperature-regulated and auto-regulated at 37°C. Also, it was down-regulated in the presence of glucose.

3.1.5. *spo0A*

spo0A is the gene that encodes the master-regulator for sporulation Spo0A in *Clostridium* and *Bacillus* species, although there are genetic differences between the two species (Burns *et al.*, 2010b; Molle *et al.*, 2003). Spo0A directly and indirectly regulates expression of several genes controlling stationary-phase events such as sporulation, DNA replication, flagellum synthesis and others (Molle *et al.*, 2003; Underwood *et al.*, 2009).

Spo0A is essential for sporulation in *C. difficile* (Underwood *et al.*, 2009). Inactivation of *spo0A* led to a completely asporogenous phenotype but did not affect growth of the bacterium. However, it did lead to reduced toxin production in the stationary phase of growth. This decrease was not because of diminished ability of cells to release toxins, indicating that *spo0A* exerted transcriptional or translational control on toxin production. The link between toxin production and *spo0A* has also been identified in other *Clostridia*. In *C. perfringens*, inactivation of *spo0A* or lower levels of transcription of *spo0A* resulted in a lack of spore and enterotoxin production (Huang *et al.*, 2004; Huang & Sarker, 2006).

As observed in *Bacillus subtilis*, *spo0A* transcription in *C. difficile* was switched on at the mid-exponential phase of growth and increased gradually during transition into the stationary phase (Fujita & Losick, 2005; Saujet *et al.*, 2011). Interestingly, this increased *spo0A* expression at the end of the exponential phase corresponded with increased expression of *tcdR*, *tcdA* and *tcdB*, indicating a transcriptional link between spore and toxin production (Saujet *et al.*, 2011).

3.1.6. Changing epidemiology of *C. difficile* in Scotland

The incidence of *C. difficile* infection in Scotland has been closely monitored since mandatory reporting was introduced in September 2006. The first surveillance report focussed on disease in patients above 65 years and identified two predominant ribotypes: 106 (64%) and 001 (18.5%) (Health Protection Scotland, 2008a). Only three cases of ribotype 027 were identified. However, by 2008, incidence of ribotype

027 disease was on the rise and even outbreaks caused by it had been reported (Health Protection Scotland, 2008b). In 2009, ribotypes 106 and 001 remained the most common strains but there was an emergence of ribotypes 027 and 078 (Health Protection Scotland, 2009a). Also in 2009, surveillance of infection in the age group from 15 to 65 years was carried out for the first time and 311 cases of CDI were reported in this group (Health Protection Scotland, 2009b). Until early 2010, incidence of infection owing to ribotypes 106, 001 and 027 remained more or less constant but towards the end of the year, prevalence of these ribotypes had decreased and an emergence of ribotypes 015, 002, 014,020 and 005 was observed (Health Protection Scotland, 2010; Health Protection Scotland, 2011). Over this short period of time, there has been a change in the predominant *C. difficile* strains in Scotland.

A look at the history of *C. difficile* in Scotland reveals that several different ribotypes were responsible for infection over the years and there has been an ongoing change in predominant strains (Taori *et al.*, 2009). Ribotype 012, to which the reference and the first-sequenced strain 630 belongs, represented 5% of *C. difficile* isolates collected between 1979 and 2004 but is no longer reported in the infected population. The incidence of ribotype 001 has increased over the years from 1.5% to 75.8% by October 2005, whereas ribotype 106 was not identified in Scottish isolates till 2004 and was responsible for 8.1% of cases by 2005 (Mutlu *et al.*, 2007). The same year witnessed the first case of ribotype 027 infection and its prevalence has increased steadily until recently (Health Protection Scotland, 2011).

The aim of this study was to identify characteristics that differentiated historic strains such as strain 630 from recently endemic ribotypes such as 027, 001 and 106. All these *C. difficile* strains are multidrug-resistant and have been associated with severe disease (Arvand *et al.*, 2009; Sundram *et al.*, 2009; Wüst *et al.*, 1982). The study aimed at measuring growth and simultaneous toxin and spore production in these strains over a 24 hour period by OD_{600} measurement, immunoassays and viable counts, respectively at the phenotypic level and by quantitative real-time PCR of the genes involved in toxin and spore production at the genetic level.

3.2. Methods

Growth of five *C. difficile* strains - strain 630, VPI 10463, ribotype 027, ribotype 001 and ribotype 106 - was studied over 10 d and 24 h (2.2.3). 24 h growth curves with sampling every 4 h was chosen as the standard methodology for all virulence factor investigations. Total toxin production was measured using a commercial combined ELISA kit (2.2.4) and spore production was measured by viable counts (2.2.5). To study transcription of the PaLoc genes and *spo0A*, a real-time RT-PCR method was developed (2.7.1) and performed (2.7.2). In order to correlate transcription of the genes to production of the individual toxins, several methods for quantification of the individual toxins were investigated. Firstly, purification of toxin A and toxin B from dialysis cultures was attempted (2.4.1-2.4.3) to obtain standards for development of immunoassays. Following the failure of these methods, commercial toxins were purchased (2.5.5). For the quantification of toxins, dot blots (2.5.1) were used and a system for protein quantification from dot blots was developed (2.5.3). However, due to the lack of sensitivity of this technique and the availability of commercial antibodies to the toxins sandwich ELISAs for the individual toxins were developed instead (2.5.6). The ELISA for toxin A was performed successfully (2.5.4) but that for toxin B was unsuccessful in detecting the toxin from culture supernatants. Thus, for toxin B, the standard cytotoxicity (and neutralisation) assay was modified to make it a quantitative method (2.6.7). Results from these experiments were collated and statistically analysed by AUC and ANOVA (2.10). Further, the *tcdC*, *tcdR* and *tcdE* genes of the strains were sequenced to identify genetic differences between them (2.3.6-2.3.8, 2.3.13). Growth-related release of virulence factors and inter-strain differences were demonstrated.

3.3. Results

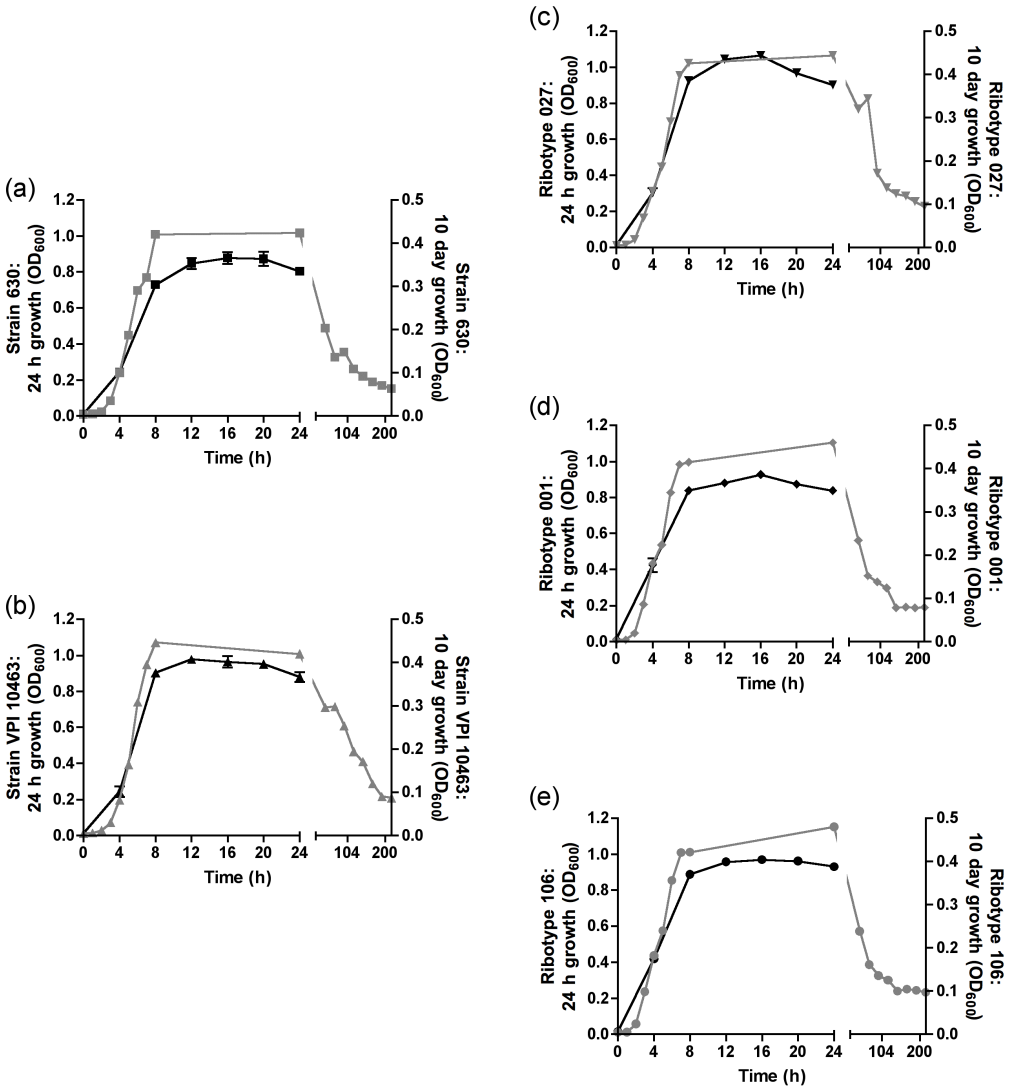
In this study, growth of five strains of *C. difficile* was studied and the simultaneous production of toxin A and toxin B, as well as spores, were analysed by phenotypic assays and gene transcription analysis.

3.3.1. Growth, total toxin production and spore production

Growth of the five *C. difficile* strains over 24 h and over 10 d both showed similar patterns (Fig. 3.2). Comparing growth of the strains individually at each time-point over the 24 h period, the only difference observed was for ribotypes 001 and 106 which showed a slight but significant increase in cell density at 4 h when compared to strains 630 and VPI 10463 ($p<0.001$) but not when compared to ribotype 027. Total toxin (A+B) production as measured by the combined ELISA kit varied significantly between the strains (Fig. 3.3). In ribotype 027, total toxin production increased significantly between 8 and 12 h. By 12 h, ribotype 027 and VPI 10463 produced significantly more toxin than the other strains ($p<0.001$). Ribotype 106 produced more toxin than 001, which in turn was greater than strain 630. All the strains produced alcohol-resistant spores and their numbers in culture increased gradually over time, except in ribotype 106 which showed a dramatic increase in spore production beyond 8 h (Fig. 3.4).

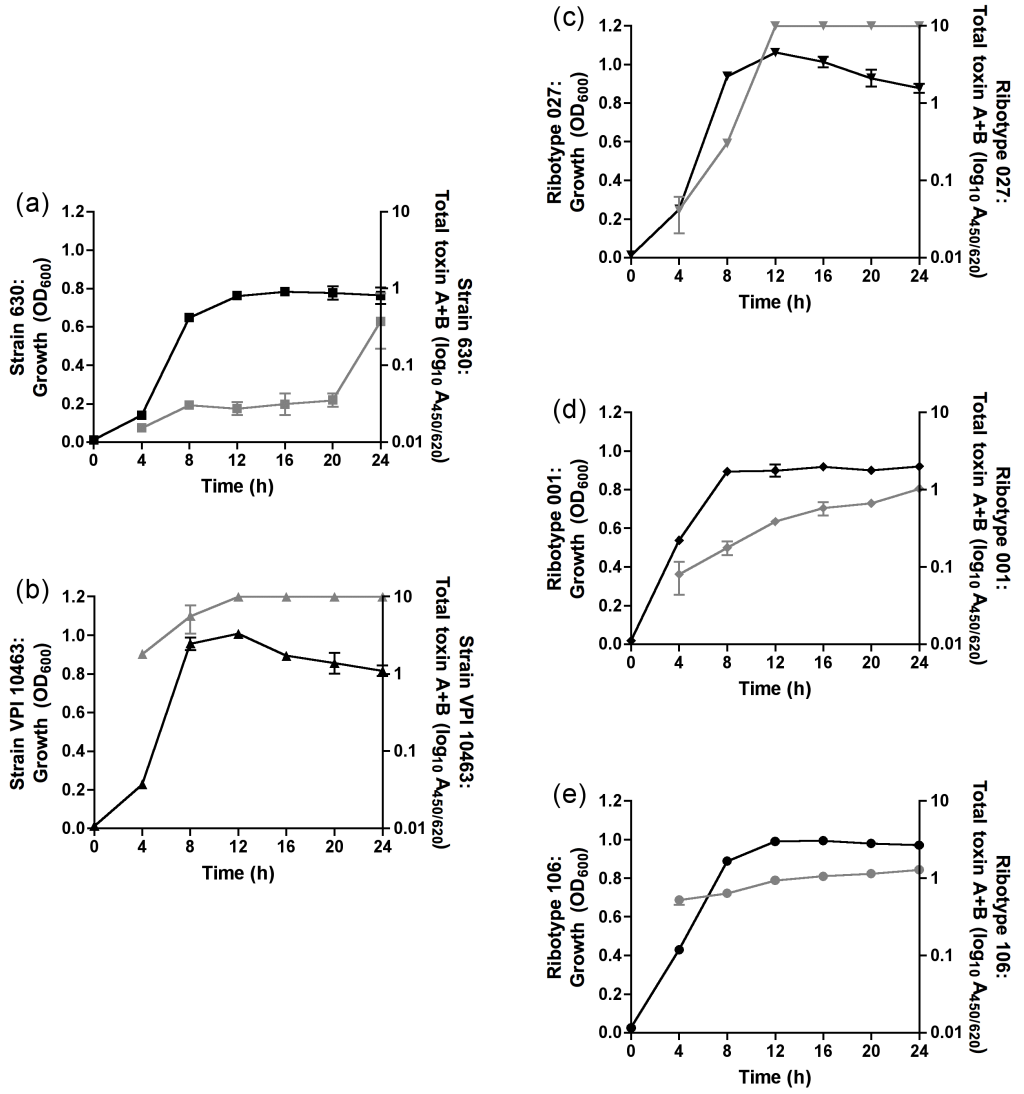
Growth, toxin production and spore production were statistically analysed (Fig. 3.5). Over the 24 h, there was only a significant difference ($p<0.05$) between growth of strain 630 and ribotype 027. In the exponential phase, this difference was observed between strain 630 and ribotypes 027 and 106, but in the stationary phase there was no difference between the strains. Total toxin production over 24 h and in the log phase of growth were significantly different between all the strains ($p<0.001$); ribotype 027 and VPI 10463 produced significantly higher amounts of total toxin. In the stationary phase, there was a significant difference between ribotype 027 or VPI 10463 and the other strains ($p<0.001$) but there was no difference between them; both strains caused a saturation of the ELISA in this phase of growth. Spore production in ribotypes 106 and 027 was significantly more than that in the other strains ($p<0.001$). The difference was less significant between strain 630 and ribotype 001 ($p<0.01$). Thus, growth rate clearly did not contribute significantly to the virulence of *C. difficile*. However, it was evident that ribotype 027 produced large amounts of toxin that could contribute to its increased virulence. Also, increased spore production in ribotypes 106 and 027 could enhance their virulence.

Fig. 3.2. Growth of five strains of *C. difficile*



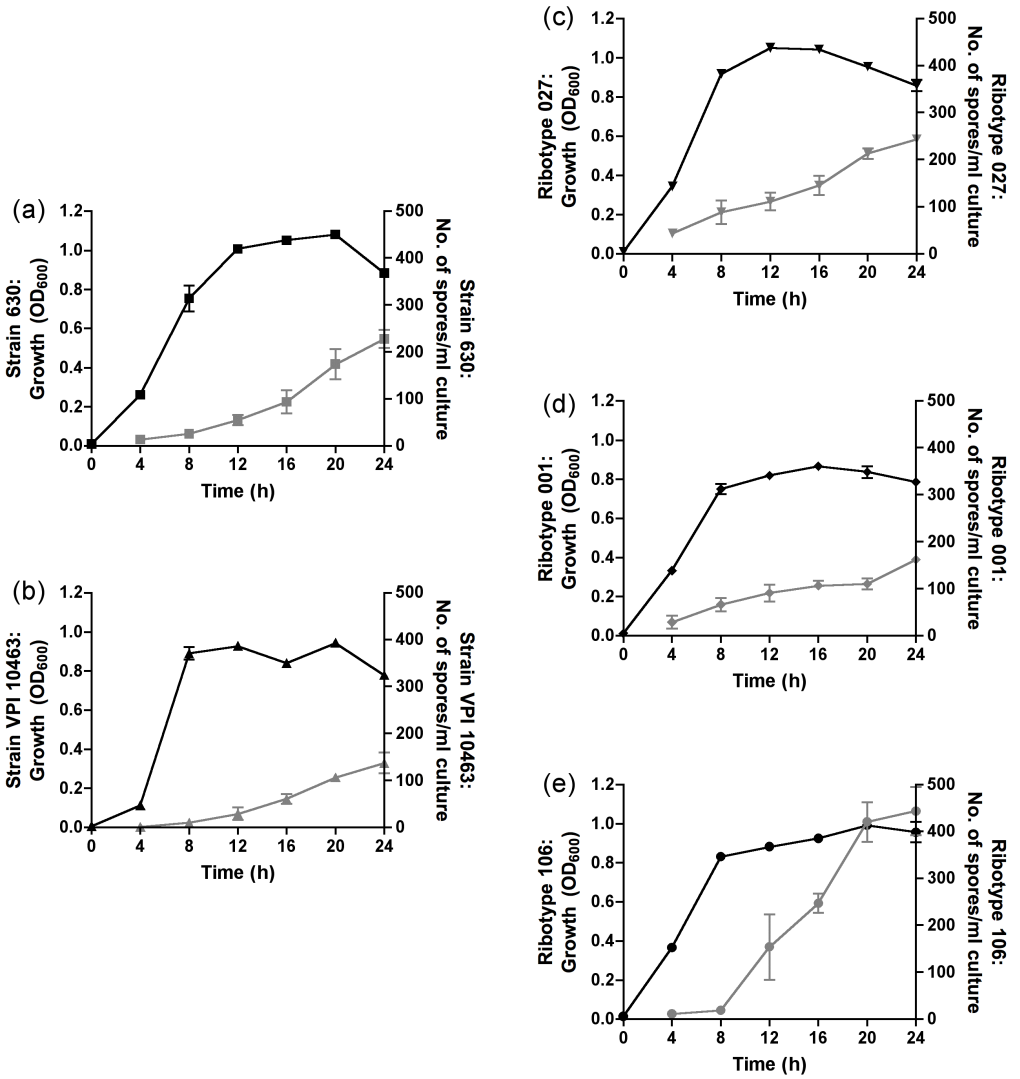
Growth of *C. difficile* (a) strain 630 (■), (b) VPI 10463 (▲), (c) ribotype 027 (▼), (d) ribotype 001 (◆) and (e) ribotype 106 (●) was studied over a period of 24 h (black) and 10 d (grey) by measuring OD_{600}. Over 24 h, the patterns of growth were similar for all the strains; however, at 4 h, ribotypes 001 and 106 showed significantly increased growth as compared to strains 630 and VPI 10463 ($p<0.001$) and ribotype 027 ($p<0.05$). This could be indicative of the behaviour of these strains *in vivo*; faster initial growth may aid colonisation. Bars indicate +/- SEM of 4 growth curves performed in triplicate each time. During the 10 d growth curve, there was no significant difference between the strains. Growth was measured once in triplicate.

Fig. 3.3. Toxin production in five *C. difficile* strains



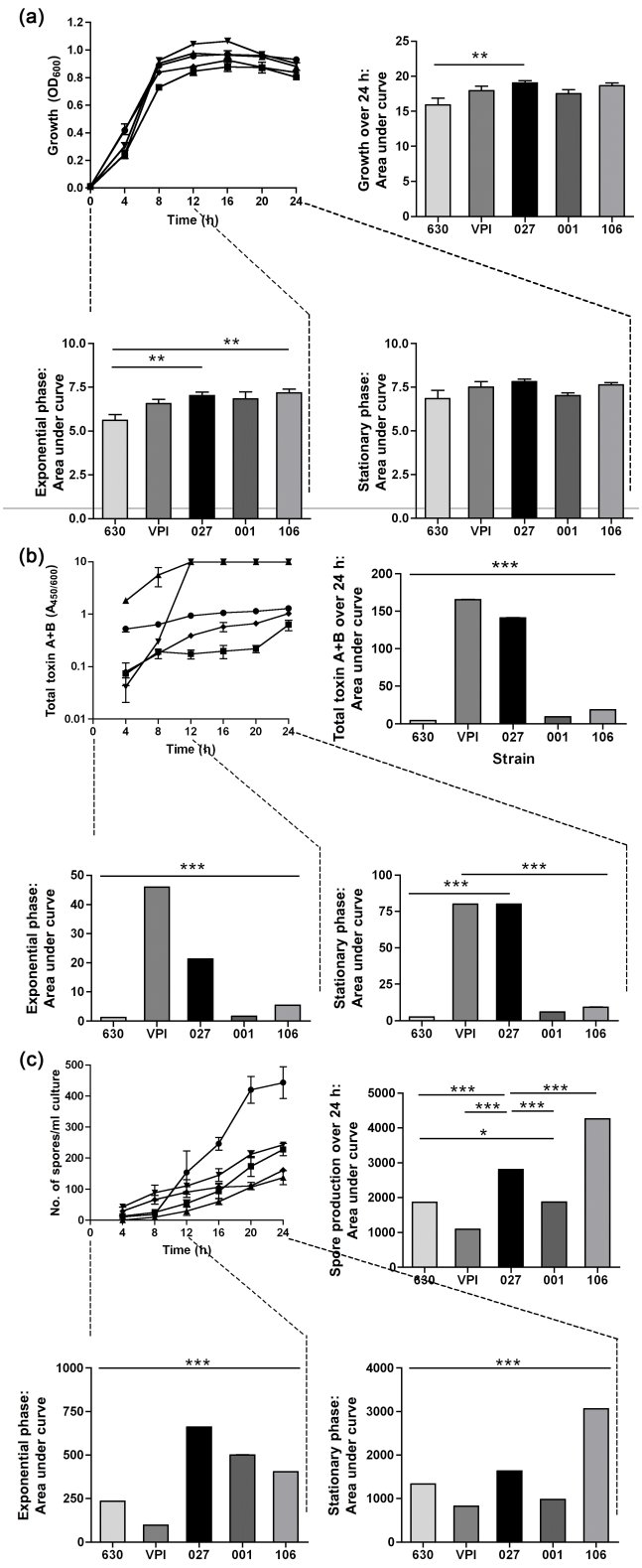
Along with growth (black), total toxin A+B production (grey) was also measured in strain 630 (a, ■), VPI 10463 (b, ▲), ribotype 027 (c, ▼), ribotype 001 (d, ◆) and ribotype 106 (e, ●) over 24 h using the *C. difficile* TOX A/B II™ kit (Techlab). In all the strains, toxin production increased over the duration of growth. By 12 h, VPI 10463 and ribotype 027 produced levels of toxin that were high enough to saturate the ELISA. VPI 10463 and ribotype 027 produced markedly greater amounts of toxin as compared to ribotypes 001 and 106, which produced more toxin than strain 630. The difference in virulence of the strains in terms of total toxin production was clearly demonstrated during the growth curves. Bars indicate +/- SEM of 3 growth curves in which each sample was tested in duplicate.

Fig. 3.4. Spore production in five *C. difficile* strains



Spore production (grey) in strain 630 (a, ■), VPI 10463 (b, ▲), ribotype 027 (c, ▼), ribotype 001 (d, ◆) and ribotype 106 (e, ●) during growth over 24 h (black) was determined by viable counts. At each time-point, 10 ml of culture was collected and treated with alcohol at a final concentration of 50%. Serial dilutions of these alcohol-resistant spores were plated in triplicate for the counts. Ribotype 106 produced the most spores and a sharp increase in these numbers was observed at 8 h. Ribotype 106 was followed by 027, ribotype 001 and strain 630 in that order. Strain VPI 10463 was the lowest spore producer as predicted. Bars indicate +/- SEM of 3 experiments performed in duplicate.

Fig. 3.5. Summary of growth, total toxin production and spore production of five strains of *C. difficile*

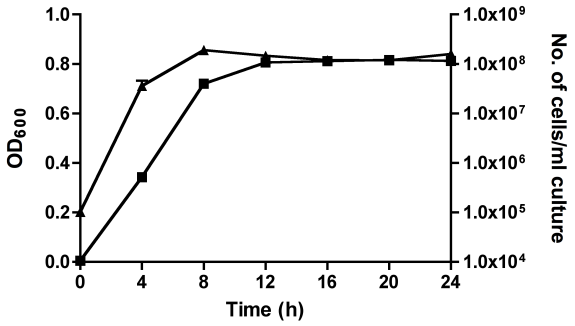


C. difficile strains 630 (■) and VPI 10463 (▲) and ribotypes 027 (▼), 001 (◆) and 106 (●) were studied. (a) Over 24 h, there was a difference in growth between strain 630 and ribotype 027 ($p<0.05$). This was observed in the exponential phase between strain 630 and ribotypes 027 and 106 ($p<0.05$) but there was no difference in the stationary phase between any of the strains. (b) Toxin production differed significantly between all the strains over 24 h ($p<0.001$). This was true for the log phase as well ($p<0.001$). In the stationary phase, there was a significant difference between the strains when compared to ribotype 027 or strain VPI 10463 ($p<0.001$) but there was no difference between them. (c) Spore production was measured by viable counts. Spore production was significantly different between the strains over 24 h ($p<0.001$) but this was less between ribotype 001 and 630 ($p<0.01$). Ribotypes 106 and 027 were the major spore producers. Bars indicate +/- SEM of 4 experiments for growth and 3 each for toxin and spore production performed in triplicate. Analysis was performed by AUC and 1-way ANOVA.

3.3.2. Development of a real-time RT-PCR to study gene transcription

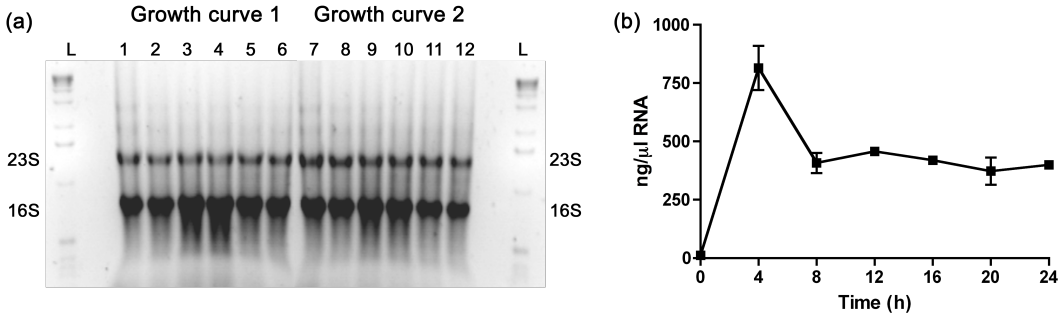
A real-time RT-PCR assay to study transcription of the PaLoc and *spo0A* in *C. difficile* was designed and standardised using strain 630. Firstly, growth was measured simultaneously by OD_{600} and viable counts (Fig. 3.6). These correlated well and volumes required to obtain $5x10^8$ cells at every time-point were estimated (50 ml at 4 h to 5 ml at 24 h). RNA was extracted from these samples and their purity was checked by $A_{260/280}$ and on an agarose gel (Fig. 3.7.a). The samples were pure but the yield of RNA decreased considerably beyond 4 h (Fig. 3.7.b).

Fig. 3.6. Growth of *C. difficile* strain 630



Growth of *C. difficile* strain 630 was measured every 4 h by viable counts (▲) and OD_{600} (■). From these, the volume of culture required to collect $5x10^8$ cells at every time-point was calculated. Bars indicate +/- SEM of 2 experiments.

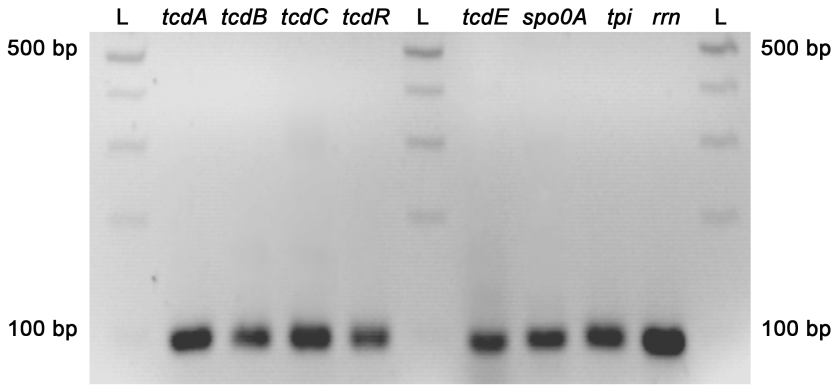
Fig. 3.7. RNA extracted from samples during growth of *C. difficile* strain 630



(a) RNA extracted from approximately $5x10^8$ cells of *C. difficile* strain 630 every 4 h was run on a 1.5% agarose gel as a preliminary purity check. The bands for 16S and 23S rRNA were visualised. Lanes 1-6 and 7-12 show RNA from samples collected at 4, 8, 12, 16, 20 and 24 h in growth curves 1 and 2, respectively. (b) Although pure RNA was efficiently extracted at all time-points, the amount of RNA extracted decreased from 4 h onwards.

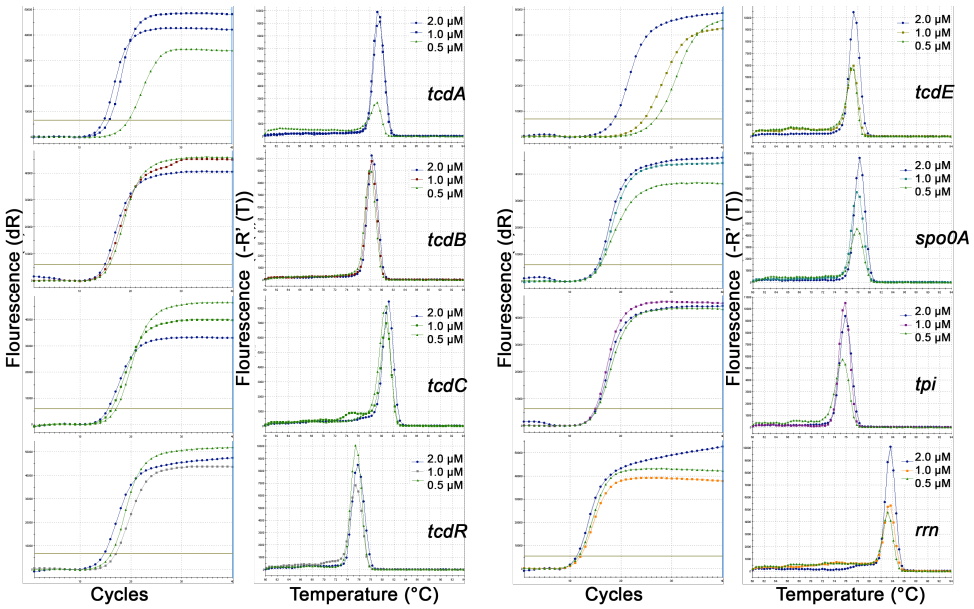
Primers designed for the assay were tested in a conventional PCR and were found to be suitable as 100 bp products were obtained for all the genes (Fig. 3.8). They also gave suitable amplification in a real-time PCR experiment (Fig. 3.9).

Fig. 3.8. Amplification of the genes of interest by conventional PCR



Primers designed for the 8 genes to be studied by real-time PCR were tested with gDNA of strain 630 in a conventional PCR. 100 bp products were obtained for all the genes.

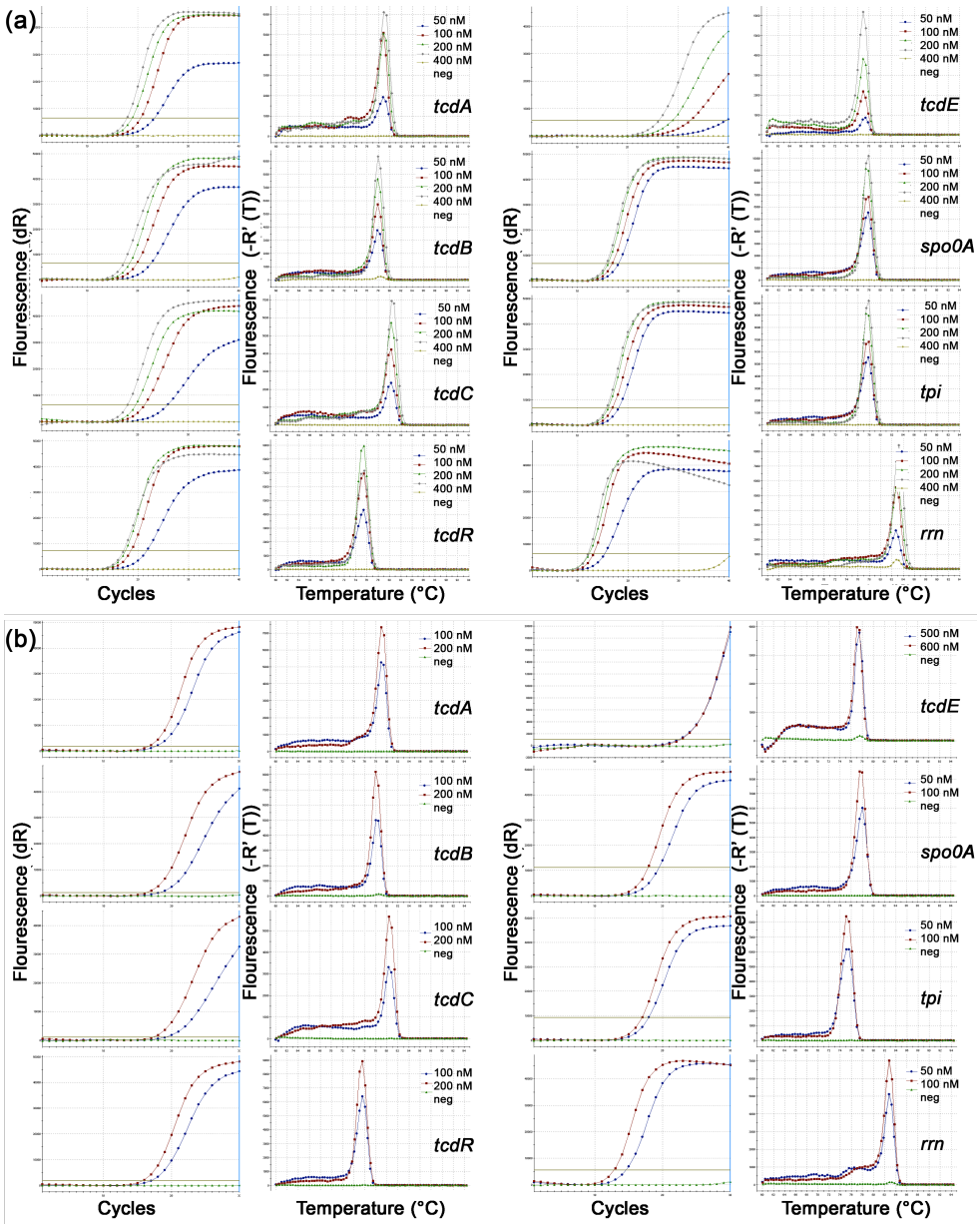
Fig. 3.9. Real-time PCR amplification of the genes of interest



All the genes in this study were suitably amplified using the primers designed when the primers were used at high concentrations in real-time PCR reactions. Products with different temperatures of melting (Tm) were obtained, which was useful to differentiate between them and detect any contamination of the primers.

After testing different primer concentrations (Fig. 3.10.a) two were selected for each gene (Fig. 3.10.b). From these, final concentrations of 200 nM of each primer were selected for *tcdA,B,C* and *R*, 500 nM for *tcdE* and 100 nM from *spo0A*, *tpi* and *rrn*.

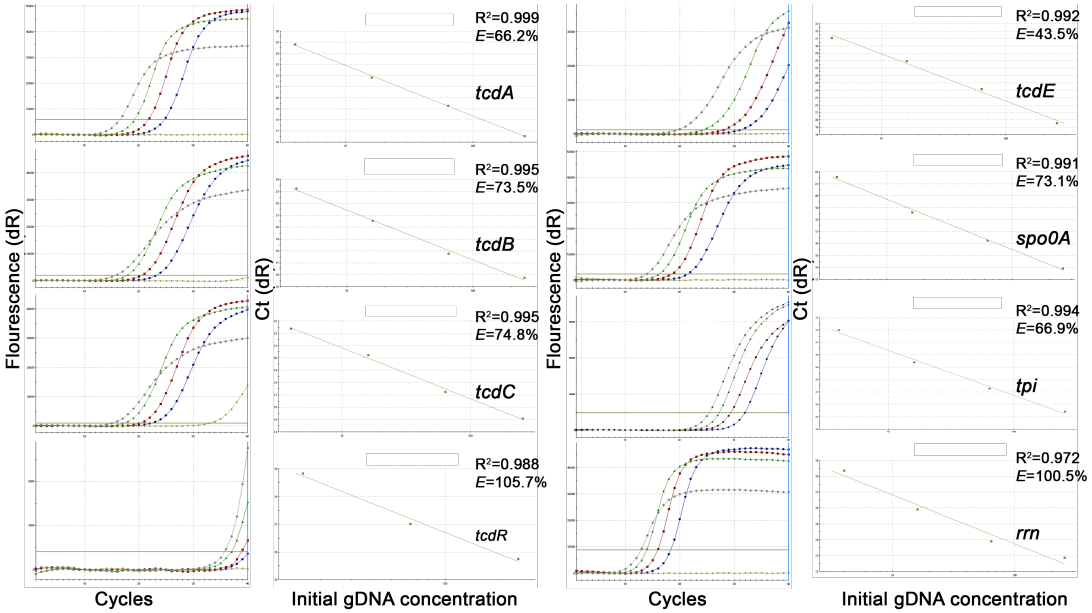
Fig. 3.10. Primer optimisation



(a) Different primer concentrations were initially selected for optimisation of the RT-PCR reactions for the different genes in this study. From the amplification obtained using these preliminary concentrations, two suitable concentrations were selected. (b) The experiments were repeated with the two selected primer concentrations to determine which was most suitable.

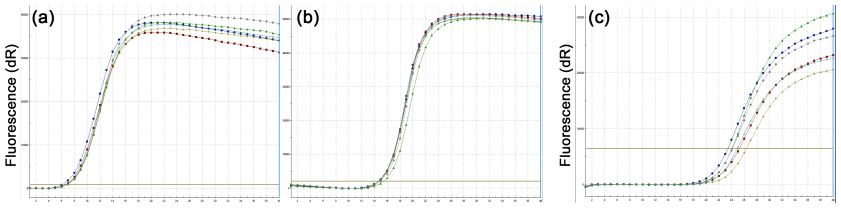
From the standard curves obtained for each gene with the selected primer concentrations, the reactions were found to be efficient and reproducible for DNA concentrations ranging from 12.5 to 800 ng (Fig. 3.11). cDNA for the genes of interest were thus diluted to an equivalent of 50 ng, but for amplification of *rrn* (Fig. 3.12.a) samples were required to be diluted to 1 ng (Fig. 3.12.b) to bring them within the range of amplification of the genes of interest. For the final assays, only *rrn* was used as a normaliser because *tpi* amplification varied over time (Fig. 3.12.c).

Fig. 3.11. Standard curves of the genes of interest with gDNA



The standard curves generated demonstrated efficient and reproducible reactions. Although the efficiency of some of the reactions was very low, they were consistent.

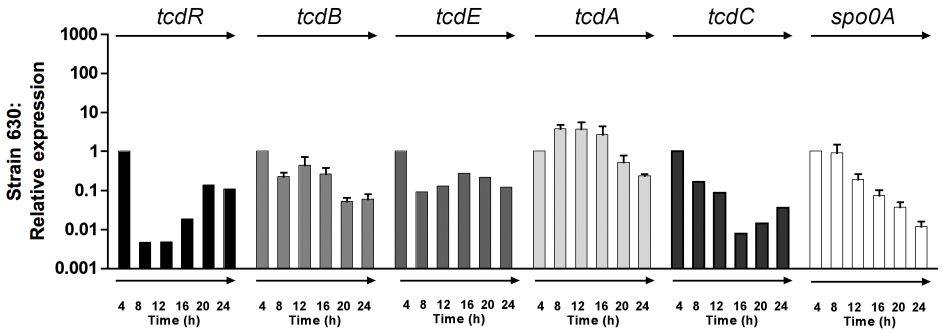
Fig. 3.12. Amplification of the housekeeping genes/normalisers *rrn* and *tpi*



(a) *rrn* amplification using cDNA diluted to an equivalent of 50 ng gave low Ct values due to early saturation of the reaction. (b) To obtain Ct values in the range of the genes of interest, cDNA was diluted to an equivalent of 1 ng of RNA. (c) *tpi* was tested but not selected as the normaliser because varied amplification was observed at different time-points.

After standardisation, a real-time RT-PCR assay was performed using cDNA prepared from the RNA extracted from *C. difficile* strain 630 cells every 4 h and the selected primer concentrations. Standard curves with dilutions of the cDNA pool were run for each gene simultaneously and gave similar results to those obtained with gDNA. The data were analysed to obtain the transcriptional trends of the PaLoc genes and *spo0A* over 24 h (Fig. 3.13). This method was then used to study gene expression in strain VPI 10463 and ribotypes 027, 001 and 106.

Fig. 3.13. Preliminary real-time RT-PCR results for *C. difficile* strain 630



Using the optimised real-time RT-PCR assay, the trends of transcription of the six genes of interest over 24 h were identified.

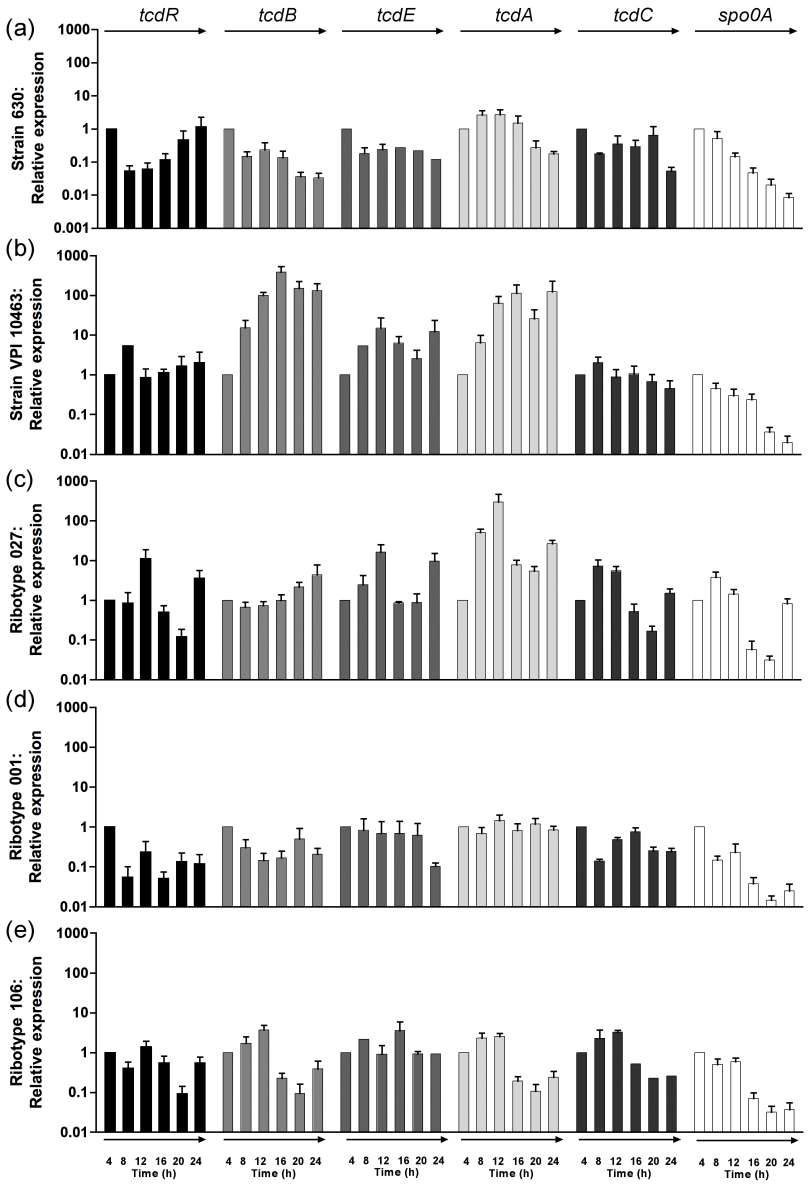
3.3.3. Transcription of the PaLoc and *spo0A*

Transcriptional analysis of the PaLoc genes and *spo0A* was successfully performed by real-time RT-PCR for the five *C. difficile* strains in this study. After standardisation, the assay was first performed for strain VPI 10463. It was observed that the transcription of *tcdA, tcdB* and *tcdR* increased over 24 h, while that of *tcdC* decreased. *tcdE* levels were found to increase till 12 h, after which they decreased, followed by a transient increase at 24 h. As these results were similar to previous observations, this method was applied to the epidemic ribotypes 027, 001 and 106 and the experiments were repeated for strain 630. Varying patterns of gene expression were observed in all the strains (Fig. 3.14).

tcdA expression increased until 12 h in ribotype 027, ribotype 106 and strain 630 and then decreased, whilst remaining almost constant in ribotype 001 over time. A similar trend was observed for *tcdB* in ribotype 106 and strain 630, although in the latter, levels were below the 4 h value. In ribotype 001, an increase in *tcdB* transcription was observed at 20 h. Interestingly, ribotype 027 was the only strain to show constantly increasing *tcdB* expression over the 24 h period studied, although it was less than that of *tcdA*. *tcdR* transcription increased steadily over time in strain 630 but in ribotypes 027, 001 and 106, peak expression was observed at 12 h followed by a decline. A transient increase in *tcdR* expression was observed at 24 h in ribotypes 027 and 106. Notably, *tcdC* transcription was similar to *tcdR* and at similar levels, contrary to the observations in VPI 10463. *tcdE* transcription increased markedly until 12 h in ribotype 027 and VPI 10463 but in the other strains expression of *tcdE* was almost steady. *spo0A* transcription decreased over time in all the strains, as expected, but in ribotypes 027 and 106 there was increasing or almost stable gene expression till 12 h, which could be the reason for their higher spore production.

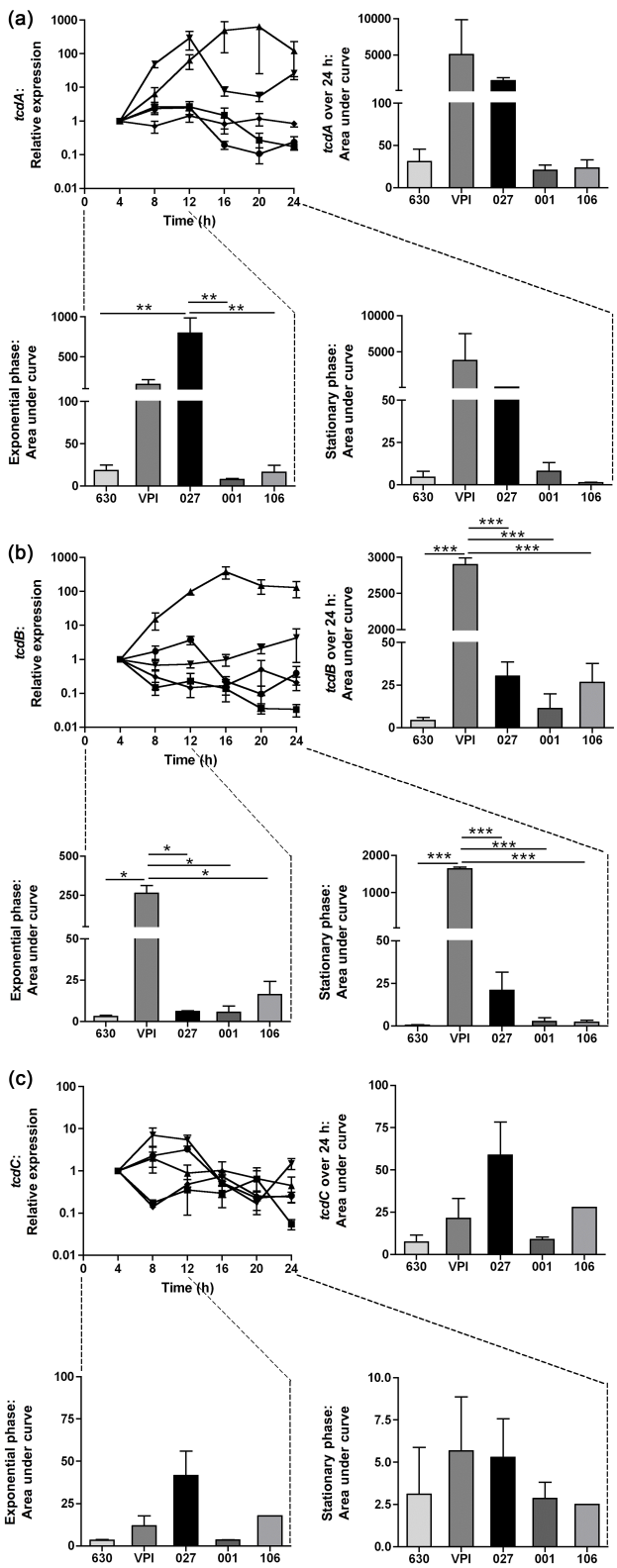
Inter-strain differences were statistically analysed (Fig. 3.15 and 3.16). In the exponential phase (0-12 h) there was a significant difference in *tcdA* expression between ribotype 027 and ribotypes 001 and 106 ($p<0.01$). *tcdB* expression was significantly different between VPI 10463 and the other strains in the exponential phase ($p<0.01$) and the stationary phase (12-24 h) ($p<0.001$). *tcdC* expression was the highest in ribotype 027 in the exponential phase of growth but there was no significant difference between the strains throughout the 24 h. Interestingly, *tcdR* expression was significantly higher in ribotype 027 and VPI 10463 in the exponential phase of growth. In the stationary phase, the difference between ribotype 027 and the other strains was significantly different ($p<0.001$) while strain 630 and ribotype 001 showed similar levels of transcription ($p<0.01$). *tcdE* expression only varied significantly in the stationary phase between the strains ($p<0.01$). The expression of *spo0A* in ribotype 027 was significantly higher in the exponential ($p<0.001$) and stationary phases ($p<0.01$). Thus, the inter-strain differences in gene expression were identified.

Fig. 3.14. Transcription of the PaLoc and *spo0A* in five *C. difficile* strains



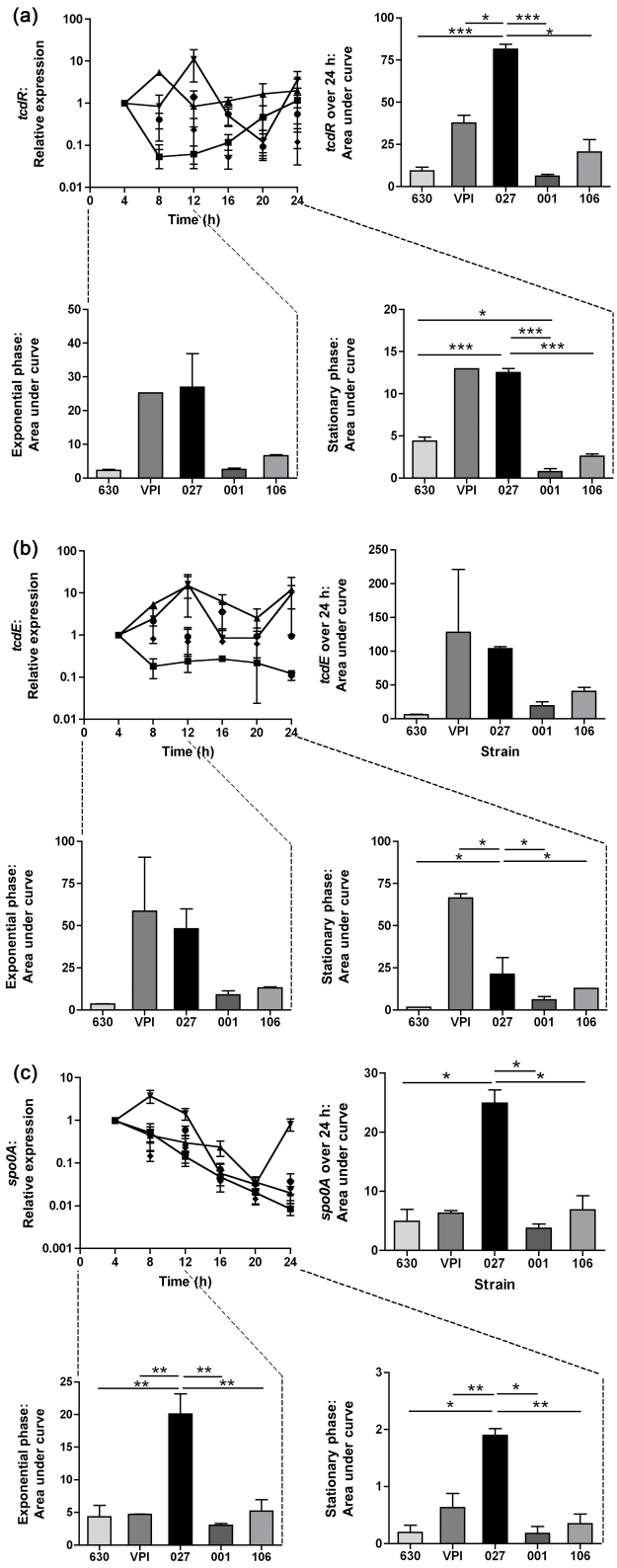
Transcription of the PaLoc and *spo0A* was studied by real-time RT-PCR in strain 630, VPI 10463, ribotype 027, ribotype 001 and ribotype 106. (a) In 630, *tcdA* and *tcdB* expression increased until 12 h and then decreased. *tcdR* and *tcdC* expression increased over time; that of *tcdE* remained constant. (b) In VPI 10463, the expression of *tcdR*, *tcdA*, *tcdB* and *tcdE* increased over 24 h, while that of *tcdC* decreased. (c) In ribotype 027, *tcdR*, *tcdE*, *tcdA* and *tcdC* transcription peaked at 12 h and then decreased. *tcdB* transcription increased over 24 h. (d) In ribotype 001, *tcdR* and tcdC transcription was similar, while that of *tcdB*, *tcdE* and *tcdA* did not vary. (e) In ribotype 106, *tcdR*, *tcdB*, *tcdA* and *tcdC* transcription peaked at 12 h, while that of *tcdE* increased until 16 h. Bars indicate +/- SEM of 6 experiments for *tcdA* and *tcdB* expression and 4 experiments for *tcdR*, *tcdE* and *tcdC* expression.

Fig. 3.15. Summary of *tcdA*, *tcdB* and *tcdC* transcription in five strains of *C. difficile*



Gene expression in strain 630 (■), VPI 10463 (▲), ribotype 027 (▼), ribotype 001 (◆) and ribotype 106 (●) was measured by real-time RT-PCR. (a) *tcdA* expression was significantly different between ribotype 027 and the other strains, except VPI 10463, but only in the exponential phase ($p<0.05$). VPI 10463 and ribotype 027 demonstrated the highest *tcdA* expression. (b) *tcdB* transcription in VPI 10463 was significantly different compared to the other strains over the entire growth curve ($p<0.001$). In the exponential phase, this difference was significant ($p<0.01$) and it was greater in the lag phase of growth ($p<0.001$). (c) Transcription of *tcdC* was highest in ribotype 027, followed by ribotype 106 and VPI 10463, but there was no significant difference between the strains in either the exponential or stationary phases of growth. Bars indicate +/- SEM of 6 experiments for *tcdA* and *tcdB* expression and 4 for *tcdC.*

Fig. 3.16. Summary of *tcdR*, *tcdE* and *spo0A* transcription in five strains of *C. difficile*



Gene expression in strain 630 (■), VPI 10463 (▲), ribotype 027 (▼), ribotype 001 (◆) and ribotype 106 (●) was measured by real-time RT-PCR. (a) *tcdR* expression was significantly different between ribotype 027 and the other strains. This difference was more significant between ribotype 027 and strain 630 and ribotype 001 ($p<0.001$) and less significant when compared to ribotype 106 and VPI 10463 ($p<0.01$). (b) *tcdE* expression in VPI 10463 and ribotype 027 was the highest, but not significantly different over the entire growth curve. There was a significant difference between ribotype 027 and the other strains in the stationary phase ($p<0.01$) (c) *spo0A* transcription was significantly higher in ribotype 027 in the exponential phase ($p<0.01$) and the stationary phase ($p<0.001$) when compared to the other strains. Bars indicate +/- SEM of 4 experiments for *tcdR* and *tcdE* expression and 6 for *spo0A*.

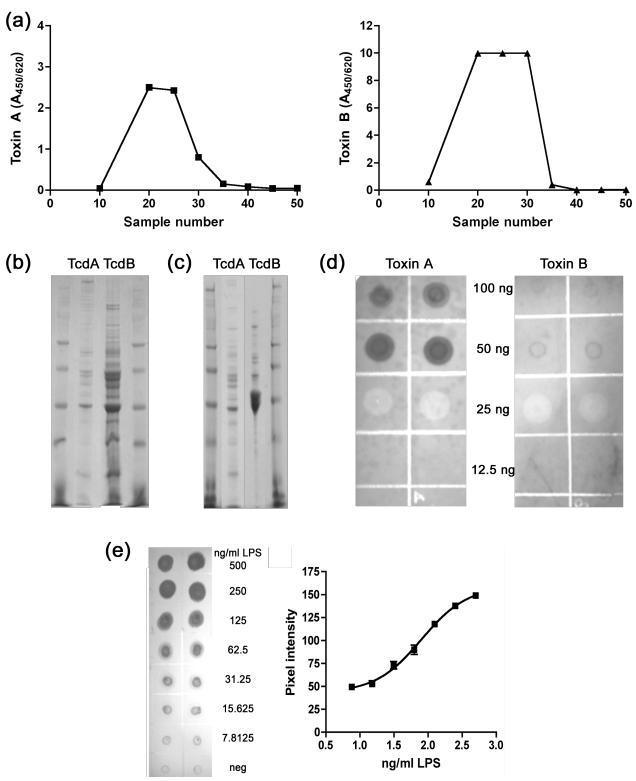
3.3.4. Toxin purification and detection

In order to correlate toxin production with individual gene expression, quantitative methods were required for each toxin. Thus, attempts were made to purify *C. difficile* toxin A (TcdA) and toxin B (TcdB) from dialysis cultures of VPI 10463. Toxins prepared by affinity chromatography (Fig. 3.17.b) and by ammonium sulphate precipitation (Fig. 3.17.c) were checked for purity on SDS-PAGE gels and several bands were observed that suggested either protein degradation or contamination, making them unsuitable as pure standards for immunoassays. When commercial toxins became available, dot blots (Fig. 3.17.d) were attempted with toxin detection using monoclonal antibodies (Table 3.1). A method to quantify proteins from dot blots using imaging software was developed with LPS (Fig. 3.17.e). However, the dot blots were insensitive to small quantities of toxin and not reproducible. Hence, there was a need for a sensitive and reproducible method for toxin quantification.

Table 3.1. Antibodies used to detect *C. difficile* toxins and their working concentrations

Method	Order	Toxin A	Toxin B
Dot blot	Primary antibody	Monoclonal antibody (Novus Biologicals, NB600-1066, Clone PCG4) 1:500	Monoclonal antibody (Novus Biologicals, NB600-1067, Clone 5A8-E11) 1:250
	Detection antibody	Anti-mouse IgG-peroxidase antibody produced in rabbit, (A9044, Sigma) 1:1000	
ELISA	Primary antibody	Polyclonal antibody (Meridian Life Science, B01245R) 1.5 µg/ml	Monoclonal antibody, (Novus Biologicals, NB600-1067, Clone 5A8-E11) 1.105 µg/ml
	Secondary antibody	Monoclonal antibody, (Novus Biologicals, NB600-1066, Clone PCG4) 0.5 µg/ml	Polyclonal antibody (Meridian Life Science, B01246R) 0.92 µg/ml
	Detection antibody	Anti-mouse IgG-peroxidase antibody produced in rabbit, (A9044, Sigma) 1:1000	Anti-rabbit IgG-peroxidase antibody produced in goat, (A6154, Sigma) 1:1000

Fig. 3.17. Initial methods employed to purify and quantify *C. difficile* toxins

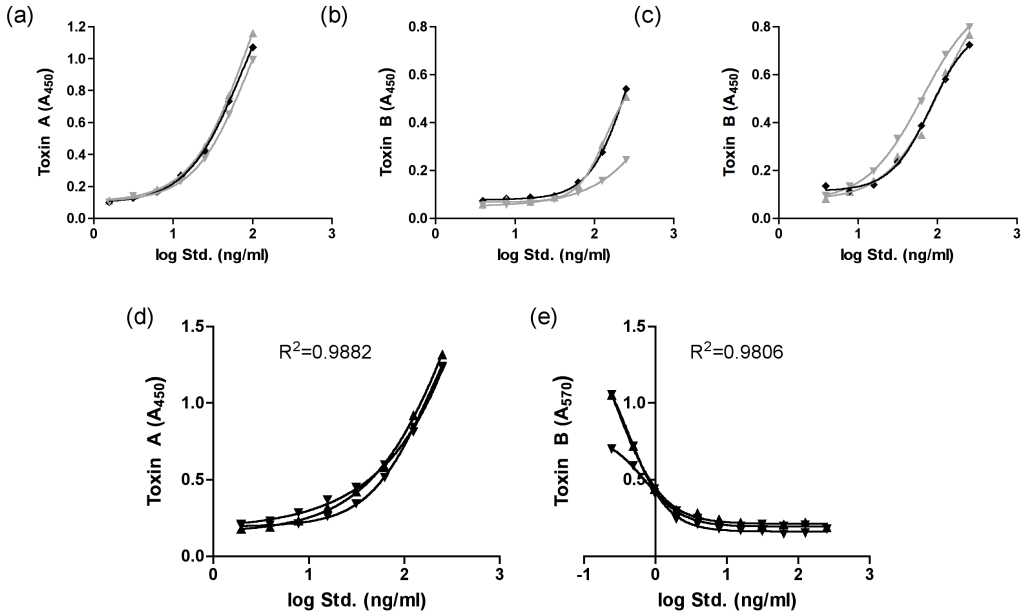


Dialysis cultures of *C. difficile* VPI 10463 were used to prepare the toxins. (a) Affinity chromatography was used to obtain toxin A. Toxins were detected using a commercial ELISA kit in 17 fractions during the elution of toxin A and in 20 samples post-elution. (b) These proteins were run on an SDS-PAGE gel and multiple bands were detected. A few faint bands above the 200 kDa mark were obtained but these could not be seen on Western blots. (c) Toxin purification was attempted by ammonium sulphate precipitation; salt concentrations of 70% and 50% were used to for toxins B and A, respectively. Dialysed proteins were then run on an SDS-PAGE gel. Multiple bands were obtained by this method too. (d) Dot blots were then attempted with commercial toxin A and toxin B and monoclonal antibodies, but the method was not found to be sensitive enough to detect the small amounts of toxin present in the early hours of *C. difficile* growth. (e) A method to quantify proteins from dot blots was developed using *E. coli* R1 LPS; the amount of protein could be quantified using pixel intensity of the spots detected in Adobe Photoshop CS.

When polyclonal antibodies for the *C. difficile* toxins became available, sandwich ELISAs were developed for both toxins using commercial standards and antibodies in Table 3.1. The ELISA for toxin A was standardised by performing a checkerboard assay using the polyclonal antibody for capture (Fig. 3.18.a). The ELISA for toxin B was similarly standardised (Fig. 3.18.b), but it was found to be more sensitive using the monoclonal antibody for capture (Fig. 3.18.c). Although the ELISA for toxin B was functional with a commercial standard, detection of the toxin from culture supernatants was unsuccessful, even in the late stationary phase of growth of VPI 10463, the strain showing the highest levels of toxin production. Attempts to alter

conditions like changing incubation times, temperatures and pH and even neutralisation of toxin A did not alter the outcome. Thus, the cytotoxicity assay was used for detection of toxin B instead; for quantification of toxin B, dilutions of the commercial standard were run in each experiment to generate standard curves, just as in an ELISA. The methods finally selected for toxin quantification were the ELISA for toxin A (Fig. 3.17.d) and the modified cytotoxicity assay for toxin B (Fig. 3.17.e). Both methods were both found to be highly reproducible and sensitive over a range of 0.5 to 500 ng/ml toxin.

Fig. 3.18. Development of an ELISA for toxin A and a cytotoxicity assay for toxin B



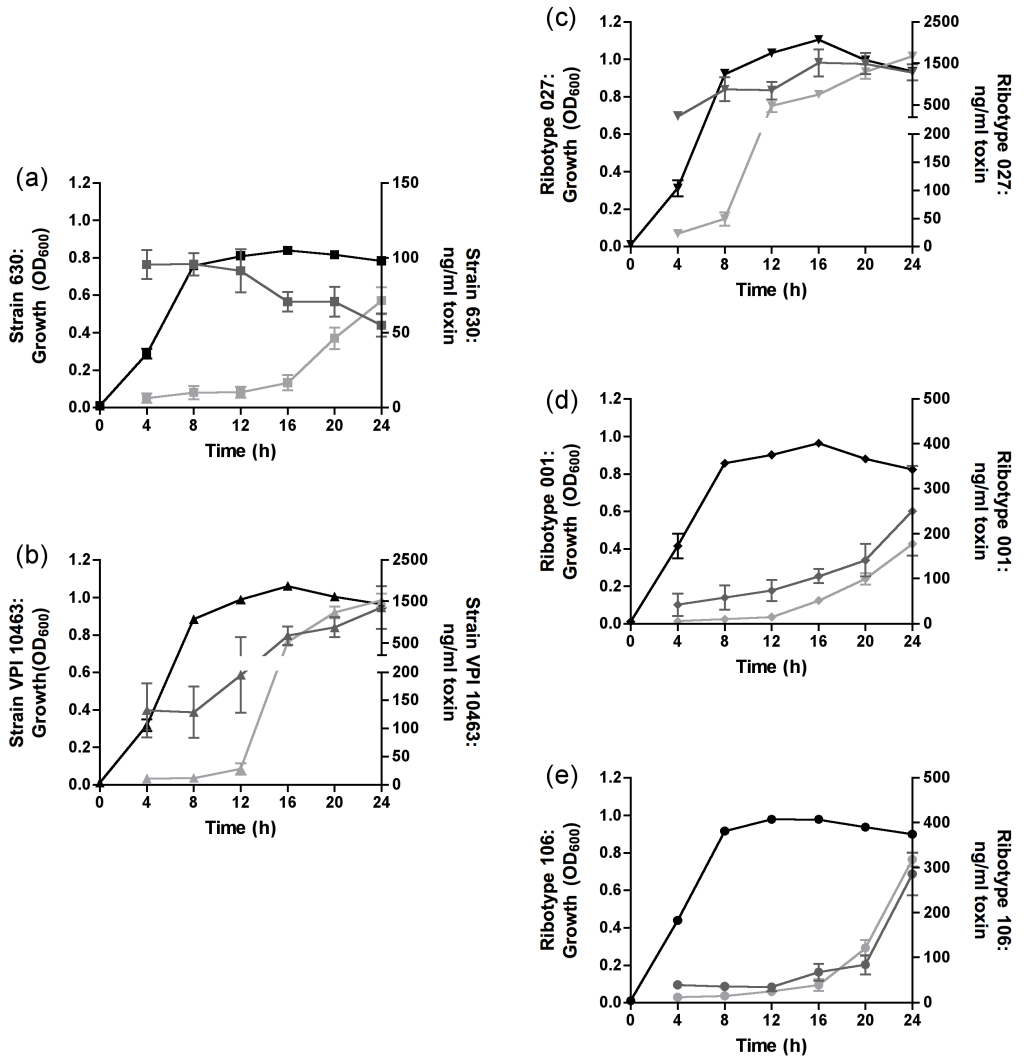
Sandwich ELISAs were developed for quantification of *C. difficile* toxin A and toxin B from culture supernatants. They were standardised by performing checkerboard assays with 2-fold dilutions of monoclonal antibodies, polyclonal antibodies and commercial standards. The polyclonal antibody was used for capture and the most suitable concentrations of both antibodies were identified from the standard curves generated (a) for toxin A and (b) for toxin B. (c) The ELISA for toxin B was found to be more sensitive when the monoclonal antibody was used for capture. When tested with culture supernatants, (d) the toxin A ELISA proved to be sensitive and reproducible; however, the toxin B ELISA was unsuccessful in detecting any toxin from supernatants. (e) Therefore, a modified quantitative cytotoxicity assay was used instead, which was also found to be highly reproducible.

3.3.5. Individual toxin A and toxin B production

Toxin A and toxin B were detected in the cultures of all strains (Fig. 3.19). Ribotype 027 produced the most toxin A up to 12 h ($p<0.001$) and the amounts increased until 24 h. All the other strains produced low levels of toxin A until 12 h. Beyond 12 h, large amounts of toxin A were detected in cultures of VPI 10463, as expected, and these were similar to those of ribotype 027. Ribotypes 106 and 001 produced toxin A at similar levels, which were significantly lower than VPI 10463 and ribotype 027 ($p<0.001$) and slightly higher than strain 630. The levels of toxin B detected in the cultures were higher than those of toxin A in all strains over time. VPI 10463 showed a steady increase in toxin B production which reached high levels at 24 h. Ribotype 027 produced significantly more toxin B than the other strains at 8 h ($p<0.01$) and this level remained almost constant until 24 h. In ribotypes 106 and 001, toxin B production increased gradually up to 20 h and then increased sharply at 24 h. Interestingly, in strain 630, toxin B production decreased over time. Differences in toxin production between the five strains were statistically compared over the different phases of growth (Fig. 3.21). Ribotype 027 produced the greatest amount of toxin A and toxin B in both the exponential and the stationary phases of growth. In the stationary phase, ribotype 027 produced significantly more toxin A ($p<0.05$) and toxin B ($p<0.001$) when compared to the other strains.

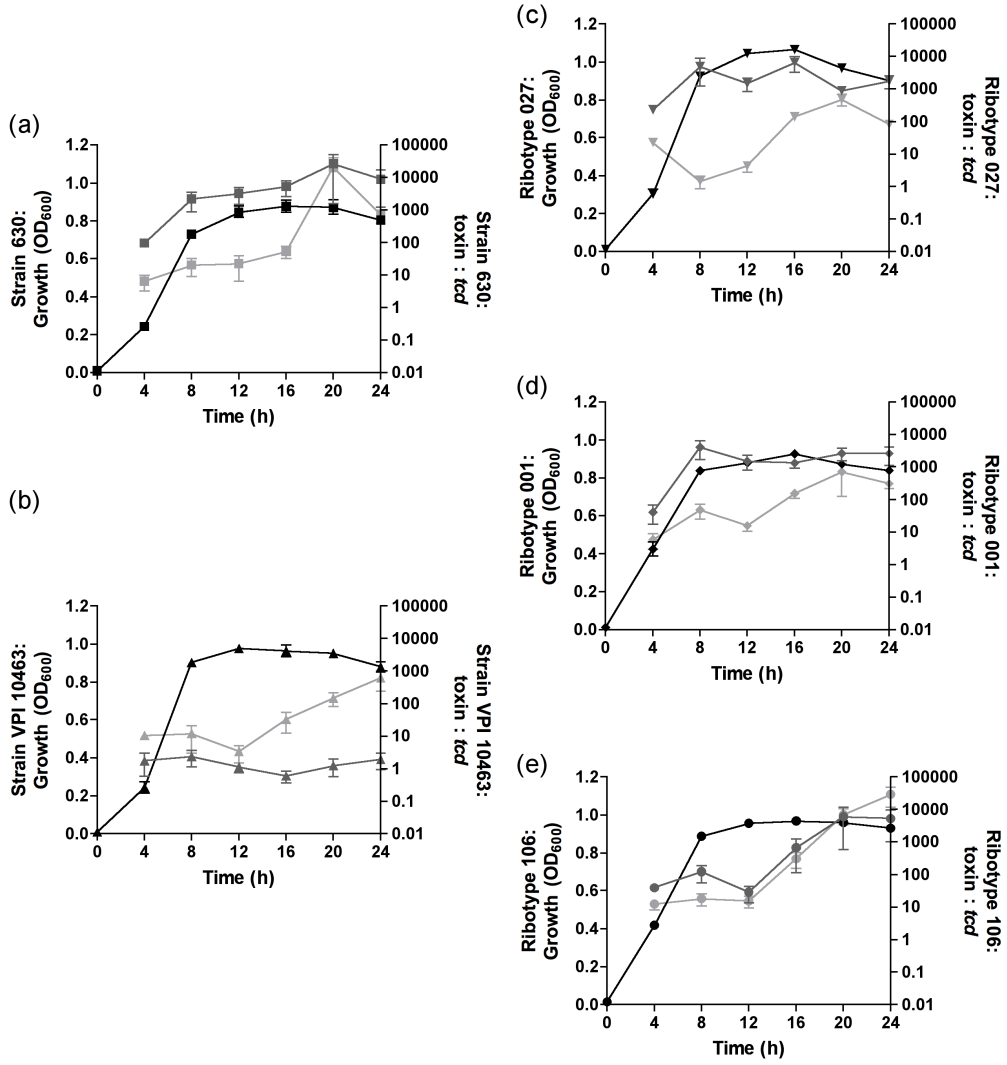
The extracellular levels of both toxins increased over time in all the strains, even when gene expression decreased and the release of toxins was thus assessed from a ratio of the amount of extracellular toxin to gene expression (Fig. 3.20); a line parallel to the x-axis indicated matched gene expression and protein amounts. For all the strains, the increased release of toxin A was evident after 12 h on entry into the stationary phase of growth. This release was most prominent in ribotype 106. For toxin B release in ribotype 106, the same pattern as toxin A was observed. In strain VPI 10463, the release of toxin B appeared to be steady over the 24 h, while in strain 630, there was a gradual release of toxin B. In ribotypes 027 and 001, there was a steady release of toxin B after the late exponential phase (8-12 h).

Fig. 3.19. Production of toxin A and toxin B in five *C. difficile* strains



Production of toxin A (light grey) and toxin B (dark grey) in the strains was studied over 24 h of growth (black) using an in-house quantitative ELISA for toxin A and the modified quantitative cytotoxicity assay for toxin B, respectively. Toxin A production increased in the strains over time. Ribotype 027 was the highest producer of toxin A up to 12 h. The levels of toxin B in the strains also increased over time, except in strain 630. Ribotype 027 and VPI 10463 produced the greatest amounts of toxin A and toxin B. Toxin B levels were higher than those of toxin A in the strains. Bars indicate +/- SEM of 6 experiments performed in duplicate.

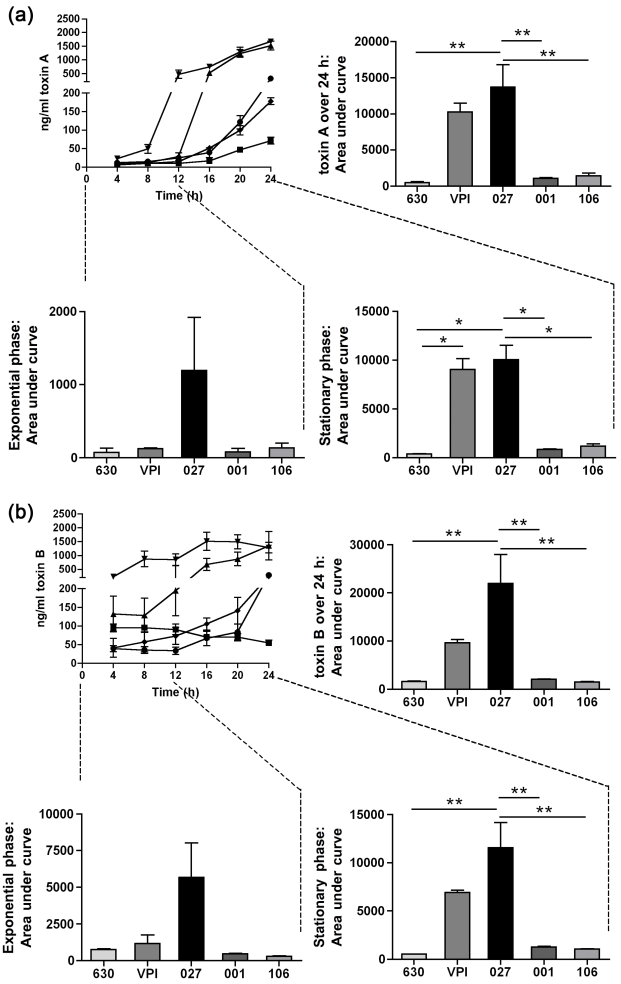
Fig. 3.20. Release of toxin A and toxin B in five *C. difficile* strains



The correlation of toxin production to toxin gene transcription was assessed by analysing ratios of toxin A value:*tcdA* expression (light grey) and toxin B value:*tcdB* expression (dark grey) at different time-points. In all the strains, the release of toxin A at 12 h is evident. For toxin B, earlier release is indicated. Bars indicate +/- SEM of 6 experiments.

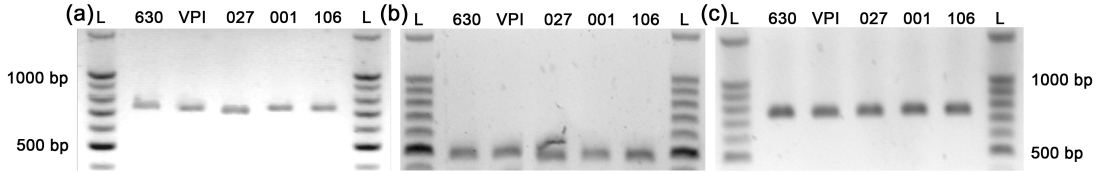
The non-toxin genes of the PaLoc were amplified by PCR (Fig. 3.22) and sequenced in order to identify any genetic differences between the strains, but no mutations were identified in any of the genes in any strain other than the *tcdC* gene of ribotype 027, which had the characteristic frameshift mutation at position 117 and the 18 bp deletion from nucleotides 330 to 347 (6.4.11).

Fig. 3.21. Summary of toxin A and toxin B production in five *C. difficile* strains



Toxin production by strain 630 (■), VPI 10463 (▲), ribotype 027 (▼), ribotype 001 (◆) and ribotype 106 (●) was measured. (a) Toxin A production was measured by ELISA. There was a significant difference between the strains and ribotype 027 over the 24 h ($p<0.05$). During the log phase, ribotype 027 produced the most toxin A and in the stationary phase this difference was significant ($p<0.01$). (b) Toxin B production was measured using a cytotoxicity assay. Over 24 h, the difference between ribotype 027 and the other strains was significant ($p<0.05$). Ribotype 027 produced the most toxin B during the log phase and this was significant in the stationary phase ($p<0.05$). Bars indicate +/- SEM of 6 experiments performed in duplicate. Analysis was performed by AUC and 1-way ANOVA.

Fig. 3.22. Amplification of the non-toxin genes of the PaLoc for sequencing



Accessory genes of the PaLoc - (a) *tcdC*, (b) *tcdR* and (c) *tcdE* - were amplified by PCR and the products were sequenced. No mutations or deletions were identified in *tcdR* and *tcdE*. In *tcdC*, the only genetic changes detected were the Δ117 and 18 bp mutations which are characteristic of ribotype 027.

3.4. Discussion

The cumulative data from this study suggest that there has been a change in the phenotype of *C. difficile* strains responsible for infection in Scotland towards increased virulence and potentially, more severe disease.

The phenotypic experiments demonstrated that although there was no significant difference in growth of the five *C. difficile* strains studied, there were differences in their respective abilities to produce toxins and spores. Growth of the five strains was similar to each other and to several previous studies (Dupuy & Sonenshein, 1998; Ketley *et al.*, 1984; Vernet *et al.*, 1989). The exponential phase of growth lasted for 12 hours, followed by the stationary phase. Total toxin production was highly diverse among the strains and ribotype 027 produced the highest levels of toxin, followed by ribotype 106. Spore production was highest in these strains, but ribotype 106 produced more spores than ribotype 027. Ribotype 001 produced less toxin and spores than ribotypes 027 and 106 but these were always more than strain 630. This is indicative of the changing epidemiology; ribotype 001 over-took strain 630 owing to enhanced toxin production and sporulation and it was then replaced by ribotype 106 and ribotype 027 which were even more toxigenic and sporogenic. The reference strain VPI 10463 produced the highest levels of toxin and least number of spores as previously observed, thus confirming that the growth conditions were suitable (Mukherjee *et al.*, 2002).

The majority of these observations are in agreement with previous work. The lack of inter-strain variability in growth confirmed the hypothesis that growth rate did not impact on virulence in *C. difficile* (Merrigan *et al.*, 2010; Warny *et al.*, 2005). They also showed a correlation between strain type and the amount of toxin produced (Wren *et al.*, 1987). However, unlike in some studies, an inverse correlation between toxin levels and spore counts was not observed; high toxin producers also sporulated more (Akerlund *et al.*, 2006), suggesting a link between the two processes (Kamiya *et al.*, 1992). Also, contrary to a study by Burns and colleagues, strain 630 neither sporulated earlier nor did it do so to a greater extent as compared to ribotype 027

(Burns *et al.*, 2010a). However, this could suggest that isolates belonging to the same ribotype could show varied sporulation (Burns *et al.*, 2010b). One of the main limitations of this study was the use of a single isolate from each ribotype.

When individual toxin production was assessed, the results showed that strains which produced more toxin A also produced more toxin B and that the ratio of extracellular toxin A:toxin B was almost 1:1. Both these observations matched with previously reported data (Mukherjee *et al.*, 2002; Tucker *et al.*, 1990; Vernet *et al.*, 1989). Only in strain 630 did toxin A levels increase over time, while those of toxin B decreased. Strain 630 has been observed to produce considerably lower levels toxin A and *tcdR* transcript than VPI 10463 and has been shown to have reduced induction of gene expression in the stationary phase of growth (Dineen *et al.*, 2007). This phenomenon could possibly explain these data. Greater toxin B production was detected earlier in the hypervirulent ribotype 027 as seen by Murray and co-workers (Murray *et al.*, 2009). Toxin release was primarily observed in the transition between the exponential and stationary phases of growth. As previously observed by others, the data presented here also suggested that ribotype 027 is capable of producing up to 20 times more toxin (A or B) than other strains (Warny *et al.*, 2005), except VPI 10463. This is a clear phenotypic advantage in this strain. Toxin production was detected in the early exponential phase and throughout the period of growth. This observation differs from studies in which glucose-containing media such as BHI were used to assess toxin production (Merrigan *et al.*, 2010). Glucose represses toxin production (Dupuy & Sonenshein, 1998). The medium used in this study was anaerobe identification medium (AIM) that does not contain any glucose. It does, however, contain cysteine but at sub-inhibitory levels. This may explain the earlier detection of toxins in this study and the greater levels detected in the stationary phase of growth.

Another interesting observation was the detection of more toxin A and than toxin B at 24 hours in all the strains, except strain 630. Toxin A is an enterotoxin with the ability to bind to epithelial cells in the gut via receptors (Krivan *et al.*, 1986; Tucker & Wilkins, 1991) and cause the initial damage by glucosylation of Rho proteins

(Aktories *et al.*, 2000). In several animal studies, it has been observed that in the absence of toxin A, toxin B is unable to induce the pathology characteristic of CDI (Lyerly *et al.*, 1985). Even in cell cultures with Caco-2 cells, it has been demonstrated that when the toxin A challenge is removed, there is no damage caused to the epithelial barrier (Sutton *et al.*, 2008). Thus, large amounts of toxin A may contribute to increased disease severity and perhaps, increased potential of ribotypes 027, 106 and 001 to induce CDI in healthy humans; greater the initial damage to the gut by toxin A, greater the chance of toxin B causing extensive cytotoxicity. Toxin B is significantly more cytotoxic than toxin A, but its role in the development of the pathology of CDI is debated. However, strains lacking the potential to produce toxin B have not yet been isolated. It has been shown that *C. difficile* strains in which toxin B was knocked-out were ineffective in colonising hamsters, contrary to studies in other systems (Lyras *et al.*, 2009). However, more recently it has been suggested that both toxins are important in CDI (Kuehne *et al.*, 2010). Whatever the importance of toxin B in disease, it is clear that the current epidemic strains produce large quantities of it. This, coupled with production of large amounts of toxin A, could explain the severity of disease associated with ribotypes 027, 106 and 001.

As observed by others, the inter-strain differences in production of toxin A and toxin B did not result from mutations or deletions in the accessory genes of the PaLoc *tcdC*, *tcdR* and *tcdE*, except perhaps the Δ117 deletion in the *tcdC* gene of ribotype 027 (Murray *et al.*, 2009). The differences were due to levels of transcription of the PaLoc genes. Greater expression of the toxin genes *tcdA* and *tcdB* in high toxin producing strains like ribotype 027 and VPI 10463 was clearly observed. Though this was not surprising, it was interesting to note the varied patterns of transcription of these genes in the other strains. Whether increasing until 12 hours and then decreasing or staying constant over the 24 hours period studied, the relationship between gene expression and toxin production was evident. However, this too differed between the toxins. For toxin A, it would appear that gene transcription peaked at 12 hours and toxin levels detected in culture thereafter were a result of accumulation. This was observed less markedly and from 8 hours onwards in toxin

B. Although transcription of *tcdB* was lower than *tcdA* in all the strains, levels of toxin B were always higher than toxin A, suggesting more efficient production of toxin B or perhaps, greater proteolysis of toxin A in culture. The latter has been suggested by other researchers (Murray *et al.*, 2009).

tcdR transcription increased steadily over time in strain 630, but in ribotypes 027, 001 and 106, peak expression was observed at 12 hours followed by a decline. A transient increase in expression was observed at 24 hours in ribotypes 027 and 106. Interestingly, *tcdC* transcription showed a similar pattern and at similar levels, contrary to the observations for VPI 10463 (Hundsberger *et al.*, 1997). *tcdC* is the negative regulator of toxin production (Dupuy *et al.*, 2008; Matamouros *et al.*, 2007) and its transcription and translation have been shown to decrease as *C. difficile* cultures enter the stationary phase of growth (Govind *et al.*, 2006; Hundsberger *et al.*, 1997). Further, the deletions found in *tcdC* in ribotype 027 have been used to explain the excessive toxin production in this strain as the truncated TcdC protein would be ineffective in preventing the complexing of TcdR with RNA polymerase (Curry *et al.*, 2007). Contrary to these studies, the data from this study showed that *tcdC* transcription increased over time, at least until 12 hours and then decreased. Although there was an evident decrease in *tcdC* expression, it did not fit into the previously described pattern, as the *tcdR* expression observed was similar and at similar levels. This suggests that *tcdC* might have a modulatory effect on toxin production, rather than a strictly inhibitory one. Another recent study has also made this suggestion (Merrigan *et al.*, 2010). Also, both toxins were detected in the exponential and stationary phases of growth, despite increasing *tcdC* expression. Others have also shown this expression of *tcdC* in both the phases of growth, although it was slightly diminished in the stationary phase (Dineen *et al.*, 2007; Karlsson *et al.*, 2008). It is possible that TcdC, being a membrane associated protein, has an effect on the release of the toxins. These hypotheses can be tested by gene knockout and protein interaction studies and would greatly add to the understanding of toxin production and release in *C. difficile*. Of note was the *tcdE* transcription in ribotype 027 and VPI 10463, the highest toxin-producing strains; it increased until 12

hours and then diminished. This increase corresponded with an increase in extracellular toxin levels observed in both these strains. The almost steady expression of *tcdE* in the other strains corresponded to a slower release of toxins. The increased *tcdE* transcription in ribotype 027 may also contribute to its hypervirulence owing to its role in toxin release (Govind & Dupuy, 2010; Tan *et al.*, 2001).

spo0A, the master-regulator of sporulation, was selected as an indicator of the magnitude of the spore producing capacity of *C. difficile* strains. Despite the expected decrease in gene expression, the trends over the first few hours were considered important in identifying inter-strain differences. Remarkably, increasing or almost stable *spo0A* expression in ribotypes 027 and 106 correlated well with their increased spore production observed at 24 hours. Thus, it is likely that increased duration of *spo0A* transcription in the early stages of growth keeps the sporulation cycle active for a longer period of time, resulting in a greater number of spores being produced and released into the environment, irrespective of environmental stresses. The link between toxin and spore production has now been described (Karlsson *et al.*, 2008; Underwood *et al.*, 2009). The increased *spo0A* transcription in ribotype 027 might also affect its increased toxin production and add to its hypervirulence.

From the data presented here, it can be concluded that *C. difficile*-ribotype 027 has several phenotypic advantages that could be responsible for its emergence as a deadly pathogen. Further, toxin B of this ribotype has been shown to be more effectively endocytosed into cells and has demonstrated toxicity to a variety of cell types in zebrafish larvae as compared to toxin B produced by strain 630, clearly indicating its increased virulence potential *in vivo* (Stabler *et al.*, 2009). However, ribotype 106 also possesses traits that explain its presence as the most common strain associated with CDI in Scotland during this study. Increased toxin and spore production in the local endemic strains has given further insight into the evolving bacterial factors affecting the epidemiology of CDI. It is likely that *C. difficile* strains are adapting to produce larger amounts of more potent virulence factors, leading to increased frequency of more severe disease.

4. Disinfectants and *C. difficile*

4.1. Introduction

C. difficile is a spore-producing bacterium and it is these spores that allow for the dissemination of *C. difficile* in the environment and facilitate the spread of disease. Thus, it is of extreme importance to successfully destroy *C. difficile* spores in order to prevent infection. To this end, a wide range several sporicidal agents have been developed but several limiting factors can diminish their efficacy and frequency of use. The effects of some commonly-used laboratory agents on different *C. difficile* strains were studied.

4.1.1. Reservoirs of *C. difficile*

C. difficile in the environment is a harmless organism (Brazier & Borriello, 2000) and the first screening of the environment led to the isolation of *C. difficile* from soil, mud, hay and animal dung (Hafiz, 1974). An extensive survey of the environment carried out in Wales showed the presence of *C. difficile* in soil and fresh waters like lakes and rivers (al-Saif & Brazier, 1996). Interestingly, it was also found in sea water, swimming pools and tap water, suggesting that the spores were resistant to salinity and common water treatment processes. More recently, *C. difficile* has been found in soil along with other clostridia, though at much lower levels (del Mar Gamboa *et al.*, 2005). Not surprisingly, vegetables, especially root vegetables, have been found to be contaminated with *C. difficile* (al-Saif & Brazier, 1996). Metcalf and co-workers isolated the bacterium from 4.5% of vegetables purchased at grocers (Metcalf *et al.*, 2010b) and Bakri and co-workers isolated it from 7.5% of packaged salads (Bakri *et al.*, 2009). Interestingly, the strains detected in these studies had been previously associated with human disease.

Animals are also a potential reservoir of *C. difficile*. Direct or indirect contact with animals and environmental contamination by animals can be responsible for a range of enteric diseases (Steinmuller *et al.*, 2006). *C. difficile* has been isolated from a variety of animals, whether diarrhoeic or not (Songer, 1996); carriage and infection

have been reported in horses, calves, pigs, chickens and even household pets (Arroyo *et al.*, 2007; Keel *et al.*, 2007; Rodriguez-Palacios *et al.*, 2006; Rodriguez-Palacios *et al.*, 2007b; Simango & Mwakurudza, 2008; Songer & Anderson, 2006). Not surprisingly, contamination of the environment of these animals has also been noted and often an overlap between human isolates, animal isolates and those from the environment has been observed (Arroyo *et al.*, 2005; Keel *et al.*, 2007; O'Neill *et al.*, 1993; Weese *et al.*, 2010a).

C. difficile has also been isolated from a variety of meat products; however, the degree of contamination is also quite varied. Reports of heavy contamination of meat products in North America include those by Songer and co-workers who isolated it from approximately 40% of all uncooked beef, pork and turkey products and even 47% of ready-to-eat products (Songer *et al.*, 2009b). Similarly, Weese and co-workers found 12% of ground beef and 71% of ground pork products to be contaminated with *C. difficile* (Weese *et al.*, 2009). They also isolated the bacterium from 12.8% of all retail chicken samples (Weese *et al.*, 2010b). Lower prevalence of *C. difficile* in meats has been reported in Europe. In a study in Austria, only 3% of mixed beef and pork products were found to be contaminated, while individual beef and pork products were free of *C. difficile* (Jöbstl *et al.*, 2010). In a similar study from France, *C. difficile* was identified at a low prevalence of 1.9% and exclusively from vacuum-packed meats (Bouttier *et al.*, 2010). Contrary to these findings, a study performed in our laboratory in which over a hundred packaged meat samples from around the UK were tested, no *C. difficile* was isolated from any of the samples. As observed in animals, the strains of *C. difficile* identified from several of these foods have been isolated from human disease. Of the five ribotypes isolated from just 1.8% of pork samples in a study in Canada, three overlapped with those involved in human disease in the region (Metcalf *et al.*, 2010a). A similar study found 20.8% of beef and 14.3% of veal samples to contain *C. difficile* strains, 25% of which overlapped with human strains (Rodriguez-Palacios *et al.*, 2007a). The presence of *C. difficile* in meat products can possibly be explained by contamination during the processing of the meats (Songer *et al.*, 2009b; Weese, 2010). It is also possible that

anaerobic conditions in vacuum packages may aid sporulation (Bouttier *et al.*, 2010). Spores have been found to survive recommended cooking temperatures (Rodriguez-Palacios *et al.*, 2010). However, the significance of their presence in food and role in dissemination is debated as the human infectious dose for *C. difficile* is yet unknown (Gould & Limbago, 2010; Weese, 2010). Further, whether animals are a source of human infection or vice versa is still unidentified; nevertheless, both are reservoirs of *C. difficile* (Gould & Limbago, 2010).

Humans are undoubtedly a reservoir of *C. difficile*; the organism was first identified as a commensal in the faecal microbiota of healthy infants (Hall & O'Toole, 1935). Since then, the asymptomatic carriage of *C. difficile* has often been demonstrated in healthy children (Donta & Myers, 1982; Holst *et al.*, 1981). Bolton and co-workers found a 30.7% carriage rate among babies within the first month, with almost half the colonised infants being faecal toxin-positive, but showing no signs of diarrhoea or colitis (Bolton *et al.*, 1984). Similarly, Collignon and co-workers observed *C. difficile* carriage in 26% of hospitalised infants and even though some of them had diarrhoea, they did not require specific treatment for CDI (Collignon *et al.*, 1993). Some investigators have also found the carriage rate in children under the age of two years to be significantly higher than in older children (Matsuki *et al.*, 2005). In one special baby unit, carriage in infants ranging from 10% to 57% was observed over a year of sampling (Taffinder *et al.*, 1997).

Asymptomatic carriage of *C. difficile* in healthy adults has been reported to range from approximately 2% to 15% (Delmée, 2001; Kato *et al.*, 2001; Nakamura *et al.*, 1981; Ozaki *et al.*, 2004; Ryan *et al.*, 2010). Both toxigenic and non-toxigenic strains have been identified from these adults. The same strain can be carried by an individual for several months or it can be replaced by a new strain or the two strains can co-exist within the host (Kato *et al.*, 2001). Carriage in hospital patients with no antibiotic history or antibiotic-associated diarrhoea has also been documented (Varki & Aquino, 1982). Asymptomatic patients can harbour epidemic strains and contribute to the nosocomial spread of *C. difficile* (Riggs *et al.*, 2007). But, perhaps

the most important reservoir is symptomatic patients in a nosocomial environment. *C. difficile* is the primary agent for pseudomembranous colitis (Bartlett *et al.*, 1978b; George *et al.*, 1978b; Larson *et al.*, 1978) but it has also rarely been identified from extra-intestinal sites. Hafiz and co-workers isolated *C. difficile* from the vaginal specimens of almost 72% of women attending a special clinic and the urethra of 100% of men with non-specific urethritis and suggested that *C. difficile* might have a role in sexually transmitted disease (Hafiz *et al.*, 1975). However, 18% of women attending a family clinic were also *C. difficile*-positive, suggesting carriage in the urogenital tract. Of the children tested, 7% had positive samples, all of whom were under the age of one. *C. difficile* has been identified as the cause of chronic osteomyelitis (Riley & Karthigasu, 1982), a splenic abscess (Saginur *et al.*, 1983), an infected lateral leg wound (Urbán *et al.*, 2010) and other diseases ranging from peritonitis to bacteraemia (Byl *et al.*, 1996; Deptuła *et al.*, 2009; García-Lechuz *et al.*, 2001). Within the GI tract, *C. difficile* has also been identified as the cause of intra-abdominal hypertension and abdominal compartment syndrome (Shaikh *et al.*, 2008).

4.1.2. Transmission of *C. difficile*

C. difficile is spread via the faecal-oral route (Lyerly *et al.*, 1988). For CDI to occur, spores or vegetative cells must be delivered to the patient's gastrointestinal tract, either by ingestion or direct inoculation via contaminated equipment (Worsley, 1998).

Symptomatic patients shed large amounts of both vegetative cells and spores into the environment (Mulligan *et al.*, 1980; Wilcox *et al.*, 2003). The environments of patients with more severe disease and frequent bowel movements were found to be more contaminated than those of patients who were asymptomatic or had mild diarrhoea (Kim *et al.*, 1981; Mulligan *et al.*, 1980; Samore, 1999). Direct contact with such an environment, in the case of patients sharing a room, was found to be a significant risk factor for the acquisition of nosocomial CDI (Chang & Nelson, 2000). The same authors also found that being a 'neighbour' in a room adjacent to a

patient with diarrhoea was a greater risk for CDI and might reflect hand-to-hand transmission of the organism by healthcare workers. In one study, 16% of samples from the rooms of patients not in isolation for CDI were found to be culture positive (Dumford *et al.*, 2009).

The transmission of CDI in the hospital can also be affected by antibiotic usage. Fenton and co-workers documented the spread of *C. difficile* to 5% of hip fracture patients in a ward within six months (Fenton *et al.*, 2008). Similarly, in another study 9.5% of previously *C. difficile*-negative patients acquired the bacterium during hospitalisation, influenced by the use of antibiotics (Rotimi *et al.*, 2002). However, duration of hospitalisation can cause carriage among patients to increase even in non-epidemic conditions (Rudensky *et al.*, 1993) and aid the dissemination of disease.

C. difficile transfer from symptomatic patients to family contacts has also been reported (Kim *et al.*, 1981; Sutphen *et al.*, 1983). Similarly, the transmission of *C. difficile* between healthy family members has also been observed (Kato *et al.*, 2001), although it was not very common. However, in the same study it was observed that cross-transmission can occur between healthy carriers in the community in clusters. The spread of *C. difficile* between asymptomatic children on the same ward has also been recorded (Larson *et al.*, 1982). Transmission outside the hospital environment is best represented by community-acquired *C. difficile* infection (Kyne *et al.*, 1998; Riley *et al.*, 1995; Wilcox *et al.*, 2008).

4.1.3. Role of surfaces in transmission of *C. difficile*

The role of the contaminated inanimate object in the spread of nosocomial disease is much debated (Hota, 2004) even though there have been reports of intensive disinfection of the environment leading to the end of an outbreak (Kaatz *et al.*, 1988). In hospitals, *C. difficile* has been isolated from a variety of items, mainly those in direct contact with a patient, including portable commodes, bathing tubes and electronic thermometers (Gerding *et al.*, 1995). Apart from objects most likely to be contaminated by faeces such as toilet seats, bedpans and scales, it was also found on floors, dust, mops and bed-linen (Fekety *et al.*, 1981; Kim *et al.*, 1981). Verity and

co-workers found the bed-frame to be the most common site from which *C. difficile* was recovered (Verity *et al.*, 2001). In one study, approximately 35% of all sampled surfaces were *C. difficile* culture-positive; commodes and toilet/sluice room floors were the sites from which *C. difficile* was most frequently cultured (Fawley & Wilcox, 2001). In another study, 4.9% of the samples yielded *C. difficile* and they were all recovered from the sluice room (Malamou-Ladas *et al.*, 1983). This difference might be representative of the different methods used for sampling; in the first study, lysozyme was added to the test medium which greatly enhanced recovery of the bacterium from the samples, whereas a germinant was not used in the latter. It is also of note that carpeted floors contaminated with *C. difficile* remained so for a longer period of time than non-carpeted hard-surface floors (Skoutelis *et al.*, 1994).

C. difficile has also been isolated from areas outside patient rooms. One study revealed the contamination of 31% of surfaces in physician work areas including telephone keypads, desktop computers, tabletops and doorknobs (Dumford *et al.*, 2009). In the same study, 10% of surfaces in nursing work areas and 21% of portable medical equipment such as medication carts, medication bar code scanners and portable computers were contaminated. Acquisition of spores from such objects has not been demonstrated and suggests that the concentration of spores outside patient rooms is lower (Donskey, 2010); nevertheless, they are contaminated. Even nursing uniforms can be contaminated with *C. difficile* (Perry *et al.*, 2001). Although the transfer of bacteria from contaminated uniforms to patients was not assessed, they are a potential source of cross-infection. An investigation into the contamination of stethoscopes showed that they were not a source of *C. difficile*, although they did harbour bacteria responsible for other nosocomial infections like coagulase-negative *Staphylococcus aureus* (Marinella *et al.*, 1997). However, in a later study *C. difficile* was isolated from 4.9% of stethoscopes (Alleyne *et al.*, 2009). As stethoscopes come into contact with the skin of multiple patients, they are likely to be sources of CDI.

Air samples have been found to be *C. difficile*-negative (Kim *et al.*, 1981; Malamou-Ladas *et al.*, 1983) but contamination of air vents has been reported (Fawley &

Wilcox, 2001). Recently, *C. difficile* was isolated from the air in an elderly care bay in the absence of an outbreak or even a confirmed case of CDI (Roberts *et al.*, 2008). The detection of *C. difficile* in the air was variable at different times of the day and interestingly, all the environmental samples tested were negative strongly suggesting that aerial dissemination was most likely, possibly due to activities such as bed-making, unloading a laundry chute or even cutting a cast, which can release large numbers of fomites in the air (Greene *et al.*, 1962a). Best and co-workers also found that *C. difficile* was isolated most frequently from the air during periods of peak activity such as ward rounds, visiting hours and patient trolley services (Best *et al.*, 2010).

Interestingly, most of the *C. difficile* strains isolated from the environment commonly matched those isolated from patients (Dumford *et al.*, 2009; Fawley & Wilcox, 2001) further strengthening the link of a contaminated environment to disease; however, which comes first is yet to be fully understood (McFarland, 2002).

4.1.4. Acquisition of *C. difficile* by healthcare workers

The exposure of healthcare workers to excretions and secretions contaminated with *C. difficile* is high and hence, they are at high risk of occupationally acquired infection, especially if they receive antibiotics (Donskey, 2004). However, some investigators found no carriage of *C. difficile* by healthcare workers (Carmeli *et al.*, 1998) despite direct contact with patients with CDI. Currently, the incidence of CDI among this group seem low; fewer than fifteen cases have been reported so far (Dorn, 2009).

Strimling and co-workers reported a case in which three nurses developed CDI from direct contact with a patient (Strimling, 1989). The nurses were all young, healthy individuals with no history of antibiotic consumption for six months before development of CDI and as environmental samples were negative for *C. difficile*, the patient was the most probable source of infection. Arfons and co-workers reported four cases of CDI in healthy individuals: a physician, a medical student, a nurse and an x-ray technician, who received short doses of antibiotics for varying conditions

(Arfons *et al.*, 2005). Hell and co-workers described the acquisition of *C. difficile* by a healthy nurse who had received oral clindamycin after a root canal, which developed into diarrhoea (Hell *et al.*, 2009). Interestingly, ribotyping revealed that the isolate belonged to ribotype 053, which was the same as that infecting three patients the nurse had been in direct contact with; two of the patient isolates were indistinguishable by multilocus variant analysis (MLVA) from that isolated from the nurse, clearly demonstrating the risk of spread of CDI from patient to healthcare worker. A similar case was also reported previously (Ray & Donskey, 2003). The most recent report of CDI in a healthcare worker involved a nurse receiving antibiotics for a sinus condition who had frequently encountered CDI patients for 10 years, but none for a month before the onset of disease (Dorn, 2009). Further, there were no outbreaks or clusters of *C. difficile* in the hospital, suggesting that *C. difficile* was acquired prior to the antibiotic treatment, which facilitated progression to diarrhoea and disease.

Contamination on the hands of healthcare workers has often been described on wards with patients suffering from CDI (Fekety *et al.*, 1981; Kim *et al.*, 1981; Samore *et al.*, 1996). In non-isolation rooms, healthcare workers generally do not use gloves and use alcohol-based products for hand hygiene (Dumford *et al.*, 2009) and there are no specific protocols and guidelines pertaining to the protection of healthcare workers from the acquisition of *C. difficile*; the guidelines are mostly aimed towards the protection of other patients (Vonberg *et al.*, 2008). It has been suggested that healthcare workers (and patients) acquire *C. difficile* from contaminated surfaces (Weber *et al.*, 2010). A correlation between transmission of *C. difficile* among patients and contamination of the hands of healthcare workers has been clearly observed (McFarland *et al.*, 1989). Further, this contamination of the hands of healthcare workers can correspond directly to the levels of environmental contamination (Samore *et al.*, 1996). In the study by Samore and co-workers, a frequently isolated strain showed the characteristics of an epidemic strain; it caused symptomatic disease, was carried by personnel and transmitted to them and patient

contacts and also contaminated the environment, thus showing the link between these factors (Samore *et al.*, 1996).

4.1.5. Laboratory-acquired CDI

Although CDI is a dreaded nosocomial infection, the risk of acquiring *C. difficile* from the laboratory has not been recognised. However, two cases of CDI caused by the hypervirulent ribotype 027 have been reported in laboratory personnel (Bouza *et al.*, 2008). The first case involved a healthy 27-year old female who suffered from severe diarrhoea which cleared without the need for antibiotics. The causative 027 strain was found to have the same antibiotype as the strains recovered from faecal samples she was previously working with. The second case involved a healthy 35-year old pregnant female who received a single dose of an antibiotic to cure a urinary tract infection. She subsequently developed severe diarrhoea and was treated with vancomycin. The 027 strain causing her CDI had the identical antibiotype to a patient 027 received by the laboratory (Bouza *et al.*, 2008).

The exposure of laboratory workers to relatively high inocula of *C. difficile* and potential highly virulent patient strains increases the risk of both acquisition and potential severity of CDI. For these reasons, the authors of the above study (Bouza *et al.*, 2008) suggested that the use of class II biosafety cabinets, the effective decontamination of surfaces, preferably with chlorine-containing disinfectants, the use of disposable gloves and proper hand-washing with soap and water must be enforced in laboratories.

The transmission of *C. difficile* among healthy individuals is possible and just as for healthcare workers, the risk of constant exposure of laboratory workers to the organism is high and hence, appropriate safety precautions and decontamination procedures must be followed in laboratories working with *C. difficile* too.

4.1.6. Need for effective environmental decontamination

Nosocomial acquisition and the environment as the main source of contamination has been clearly demonstrated (Delmée *et al.*, 1988; Kim *et al.*, 1981). The need to

reduce the bacterial load on surfaces is evident, but whether disinfectants are essential for this purpose is debated as surfaces come into contact with intact skin which provides a barrier (Rutala & Weber, 2001). However, regular cleaning and disinfection must be performed as a precautionary measure in addition to others such as the use of disposable equipment and stringent hand-washing must be implemented alongside surface disinfection (Testore *et al.*, 1988).

Several reports regarding the control of outbreaks by effective decontamination exist. Kaatz and co-workers demonstrated that the use of an unbuffered hypochlorite solution (500 ppm available chlorine) reduced environmental contamination by 79% (Kaatz *et al.*, 1988) and resulted in the end of an outbreak. They also showed that buffered hypochlorite (1600 ppm available chlorine) could bring about a 98% reduction in environmental contamination. In another study, the use of a glutaraldehdye-based disinfectant on a ward with 10 CDI patients helped to gradually control an outbreak without the isolation of patients (Testore *et al.*, 1988). Cartmill and co-workers observed that the use of a 1:1000 hypochlorite solution for regular surface cleaning resulted in negative environmental samples (Cartmill *et al.*, 1994). They also found that deep cleaning of wards, which involved clearing it of all curtains and linen and washing the floors and walls, decreased the environmental burden of *C. difficile* to below detectable levels. Similarly, in a university-based hospital, cleaning of patient rooms and environmental areas with unbuffered 1:10 hypochlorite led to a 50% reduction in CDI rates after two months (Apisarnthanarak *et al.*, 2004). Mayfield and co-workers witnessed a significant reduction in the incidence of CDI when quaternary ammonium compounds were replaced with hypochlorite for cleaning and disinfection (Mayfield *et al.*, 2000). A similar observation by McMullen and co-workers demonstrated the benefits of hypochlorite solution in endemic and hyper-endemic situations (McMullen *et al.*, 2007). The cross-contamination of serial patients in the same room and between patients in adjacent rooms has also been controlled by extending hypochlorite treatment to all lateral surfaces and areas within and outside of patient rooms (Whitaker *et al.*, 2007). Even just terminal cleaning of rooms with hypochlorite (5000 ppm available

chlorine) has been shown to reduce CDI incidence by 48% (Hacek *et al.*, 2010). Besides chlorine-based agents, hydrogen peroxide vapour treatment has also been proved to be effective in completely eliminating environmental contamination by *C. difficile* and decreasing the incidence of CDI (Boyce *et al.*, 2008). Thus, the importance of surface decontamination in reducing the dissemination and acquisition of *C. difficile* is unquestionable.

Although the need to disinfect high-contact surfaces is obvious, the disinfection of floors is highly debated as it is thought that they play a negligible role in disease transmission (Favero & Bond, 2001). In one study, it was found that when certain types of disinfectants (oxygen- and aldeyhde/alcohol-based but not quaternary ammonium compounds) were used to clean floors, there was a reduction in the total bacterial count, but when only detergent was used, there was introduction of a greater bacterial load into the environment (Dharan *et al.*, 1999). Despite the clear efficacy of disinfectants in reducing environmental contamination, there was no difference in the incidence of infection in either treatment. The results from a separate but similar study also showed that the disinfection of floors was unnecessary and had no impact on the infection rate; although insignificant, the use of detergent alone was linked with lowered incidence of disease (Danforth *et al.*, 1987).

An argument in favour of floor disinfection was made by Exner and co-workers (Exner *et al.*, 2004). In an experimental model mimicking the cleaning of floors using a mop they demonstrated that the use of detergents was as ineffective as using water alone. Further, there was a spread of bacteria from the area of contamination to clean areas. The authors thus suggested that disinfection must be part of a holistic approach for hospital hygiene.

The use of disinfectants on floors is considered to be an added expense with no scientific backing (Rüden & Daschner, 2002). Also, the side-effects associated with such use makes them less appealing to housekeeping staff. Moreover, detergent cleaning produces shiny floors which are more visually pleasing (Danforth *et al.*, 1987). However, the use of a single cleaning strategy could ensure uniformity in

cleaning and training procedures (Rutala & Weber, 2001). Voss and co-workers suggested that disinfection of floors as routine practice is not necessary, but must be performed if the situation demands it (Voss *et al.*, 2003); contamination of floors with body fluids and faecal matter would be such conditions (Rutala & Weber, 2001). Thus, floor disinfection is still debatable but that of high-contact areas in preventing the spread of nosocomial disease is not.

4.1.7. Survival of spores and vegetative cells

Vegetative cells and spores of *C. difficile* have different susceptibilities to a variety of conditions, including nutrients (Buggy *et al.*, 1983); the presence of taurocholate in agar improves the recovery of spores but has no effect on the recovery of vegetative cells. Using pre-reduced media had the same effect. However, it has been observed that when *C. difficile* strains were grown in faecal emulsions for 72 hours and aliquoted and stored, the recovery of vegetative cells and spores was not affected by storage at 4°C or -20°C (Freeman & Wilcox, 2003) which could be a result of the strains being in a buffered environment. These distinguishing characteristics are important in the recovery of *C. difficile* when estimating the levels of environmental contamination. However, the key differences in the survival of vegetative cells and spores within the host and in the environment are of greater significance.

Environmental shedding of *C. difficile* commonly persists at the time of resolution of diarrhoea of a patient and can continue after therapy (McFarland *et al.*, 1989; Mulligan *et al.*, 1980; Sethi *et al.*, 2010). Even healthy individuals treated with antibiotics shed large amounts of *C. difficile*, which is reversed at the end of treatment (Chachaty *et al.*, 1993). The number of vegetative cells in the stool of patients before antibiotic treatment for CDI is significantly higher than after treatment has begun (Jump *et al.*, 2007). Following treatment, mainly spores are recovered from the stool of patients, demonstrating the resistance of spores to antibiotics that clearly eliminate vegetative cells. Further, within the host, spores are able to survive the low pH of the gastric contents of patients who are not on acid-suppressive therapy, while vegetative cells cannot. The survival of vegetative cells

increases in gastric contents as the pH increases, with significant survival above pH 5 (Jump *et al.*, 2007). Thus, the internal environment of the host may influence the number of vegetative cells and spores released into the environment.

Vegetative cells of *C. difficile* in suspension dried onto surfaces such as glass in five to 10 minutes and could only be recovered for up to 15 minutes (Buggy *et al.*, 1983). On the other hand, the recovery of spores of *C. difficile* was unaffected even after overnight drying of the suspension on the surface. However, it was later shown that the reason for the lack of recovery of vegetative cells from surfaces was that they died of desiccation (Jump *et al.*, 2007). Vegetative cells have been found to survive on moist surfaces at room temperature under aerobic conditions for 3 hours, 6 hours and sporadically up to 12 hours (Jump *et al.*, 2007; Weber *et al.*, 2010). In their studies, Jump and co-workers showed that under anaerobic conditions, vegetative cells survived for 12 to 24 hours; spores, being more robust, survived for up to 24 hours on dry or moist surfaces (Jump *et al.*, 2007). *C. difficile* spores can persist on hospital floors for several months; artificial contamination of the floor of an unused hospital room and subsequent sampling revealed that even though the number of organisms recovered declined tremendously during the first few days of sampling, *C. difficile* could still be isolated from the floor for five months (Kim *et al.*, 1981).

4.1.8. Decontamination strategies for *C. difficile*

The need for agents to control the dissemination of *C. difficile* led researchers to test the efficacy of various chemical agents against it, especially its spores. Some of the earliest disinfectant studies against clostridial spores employed glutaraldehyde (Kelsey *et al.*, 1974). At a concentration of 2%, glutaraldehyde was able to kill spores of *C. difficile* in 10 minutes, while at 0.2%, 30 minutes were required (Dyas & Das, 1985); dilutions much less than these were unable to inactivate spores (Rutala *et al.*, 1993a). However, a study investigating two glutaraldehdye-based disinfectants found that up to 4 hours of direct contact could be required for a 4 $\log_{10}$ reduction in spore counts (Horejsh & Kampf, 2011). On the other hand, at a concentration of 2%, the oxygen-releasing compound they tested was able to bring about a 4 $\log_{10}$

reduction in one hour; as the concentration was decreased, the time required for the same reduction increased. Active oxygen-based agents have been found to be significantly more effective than quaternary ammonium compounds in disinfecting bathrooms, toilets and furniture in a healthcare centre (Dharan *et al.*, 1999). Glutaraldehdye can cause dermatitis and asthma in users (Wullt *et al.*, 2003b) and thus, is not preferable for long-term usage.

Sodium hypochlorite (bleach, NaOCl) has been one of the most widely tested disinfectants against *C. difficile*, not only in the laboratory but also in hospitals. Bleach at 5000 ppm is the most suitable to obtain a 6 $\log_{10}$ reduction in *C. difficile* spores in 10 minutes, with 3000 ppm requiring 15 minutes and 1000 ppm requiring 15 to 25 minutes for the same effect (Perez *et al.*, 2005). Even when tested on spores dried on stainless steel, 5000 ppm bleach had the same effect (Omidbakhsh, 2010). The efficacy of 5000 ppm bleach could be further enhanced by spraying the bleach onto the surface, allowing contact of 3 minutes, followed by wiping the surface with a cloth dipped in the same bleach (Alfa *et al.*, 2010). However, due to the quick drying of bleach and the difficulty in spreading an even concentration over a surface, less cleaning and disinfection might be achieved (Omidbakhsh, 2010). Also, the use of bleach at concentrations of 5000 ppm requires the use of special personal protective equipment (PPE) (Alfa *et al.*, 2010). A formulation of super-oxidised water that releases hypochlorous acid and free chlorine radicals can eliminate spores in five minutes, but its efficacy decreases in the presence of organic matter (Shetty *et al.*, 1999). Thus, pre-cleaning is required but it is non-toxic and non-corrosive. A similar anolyte investigated by Robinson and co-workers was able to bring about a 2.5 $\log_{10}$ reduction in spores within 10 seconds of treatment and was able to inactivate spores below the level of detection in 20 seconds (Robinson *et al.*, 2010). Being effective, cheap and environmentally compatible, these electrochemically activated solutions might be the next step in hospital disinfection.

Sodium dichloroisocyanurate (NaDCC) agents are considered to be superior disinfectants to bleach, especially in the presence of organic matter (Bloomfield &

Uso, 1985). The use of one such disinfectant was able to reduce the incidence of CDI cases on a hospital ward where environmental contamination corresponded to incidence rates (Wilcox *et al.*, 2003). However, in one investigation it was observed that though bleach was superior to NaDCC against vegetative cells, they were both equally sporicidal (Fawley *et al.*, 2007). Under clean conditions, an agent containing 1000 ppm NaDCC and a detergent required just three minutes for a 3 $\log_{10}$ reduction in spores of ribotype 027 seeded onto stainless steel, but contact of 9 minutes was required to eliminate spores to levels below the detection limit of approximately 5 $\log_{10}$ (Wheeldon *et al.*, 2008b). This efficacy was further reduced in the presence of organic matter; 30 minutes were required for a reduction greater than 3 $\log_{10}$. Ungurs and co-workers observed that 1000 ppm of NaDCC gave a minimal reduction in spore load even after two hours, but increasing the concentration to 6000 ppm resulted in complete decontamination in 20 minutes (Ungurs *et al.*, 2011). Wiping the surface with the agent increased cleaning efficiency and pre-cleaning with detergent was even more superior as soil tends to quench the effect of NaDCC.

Peracetic acid is also sporicidal. At a concentration of 10% it was found to be less efficient than NaDCC, but it is less harmful to personnel (Wheeldon *et al.*, 2008b). Its drawback however, is that 30 to 60 minutes could be required for inactivation of spores. In a different investigation, it was observed that a peracetic acid disinfectant could inactivate 99.9% of spores within 15 minutes and could cause a 4 $\log_{10}$ reduction within five minutes despite soiling (Wullt *et al.*, 2003b). Contrary to the results of Wheeldon and co-workers, Block observed that peracetic acid was better than NaDCC (Block, 2004). It completely disinfected stainless steel contaminated with *C. difficile* spores in 10 minutes, but was less effective on polyvinyl chloride.

Accelerated hydrogen peroxide (AHP) compounds can also inactivate *C. difficile* spores in 10 minutes (Perez *et al.*, 2005) as effectively as bleach at 5000 ppm and even produce a 2 to 3 $\log_{10}$ reduction in surface-dried spores in one minute (Alfa *et al.*, 2010). AHP disinfectants performed better than stabilised hydrogen peroxide disinfectants (SHP) (Alfa *et al.*, 2010). An AHP gel could kill 6 $\log_{10}$ dried spores

within 10 minutes. Further, it formed a uniform layer over the surface due to its viscosity and took 10 minutes to dry, thus ensuring that the required contact time for sporicidal activity was achieved before the surface was visibly dry (Omidbakhsh, 2010). A hydrogen peroxide dry mist system for the contamination of entire rooms was recently tested. Shapey and co-workers found that environmental contamination by *C. difficile* could be reduced by 94% with this system and it was effective against the three main UK epidemic ribotypes 106, 001 and 027 (Shapey *et al.*, 2008). Similar results were obtained in another study in which the hydrogen peroxide dry mist system achieved a 91% reduction in contamination as compared to the 50% reduction obtained by hypochlorite cleaning (Barbut *et al.*, 2009b). Ozone (25 ppm) as a gaseous sterilant has also been tested and found to be effective in bringing about at least a 4 $\log_{10}$ reduction in *C. difficile* spores (Sharma & Hudson, 2008).

More recently, alternatives to chlorine- and oxygen-releasing agents have been sought. Acidified nitrite was tested for its sporicidal activity against *C. difficile* and was found to be effective and also unaffected by the presence of organic matter (Wullt *et al.*, 2003b). Being harmless to users or surfaces, it is a practical sporicide but must be freshly prepared for optimum activity. Metals as sporicidal agents have also been studied. Gant and co-workers studied three copper-based biocides which achieved a 2 to 3 $\log_{10}$ reduction in *C. difficile* spores in 30 minutes (Gant *et al.*, 2007). Surface cleaning using ultramicrofibre cloths dipped in these agents was also effective at decontaminating surfaces seeded with ribotype 027 spores. Metallic copper is bactericidal, but not sporicidal (Wheeldon *et al.*, 2008c). However, when the spores were exposed to 1% sodium taurocholate, a 3 $\log_{10}$ reduction resulted in three hours, which was unaffected by soiling. Triggering germination by treating surface-dried spores with a solution containing sodium taurocholate also increased their susceptibility to UV-C radiation (Nerandzic & Donskey, 2010). A UV-C device has also been tested for its ability to decontaminate rooms (Nerandzic *et al.*, 2010). A 2 to 3 $\log_{10}$ reduction in spores was achieved in approximately 45 minutes when the device was tested with contaminated surfaces and when used in rooms, an 80% reduction in positive samples was obtained.

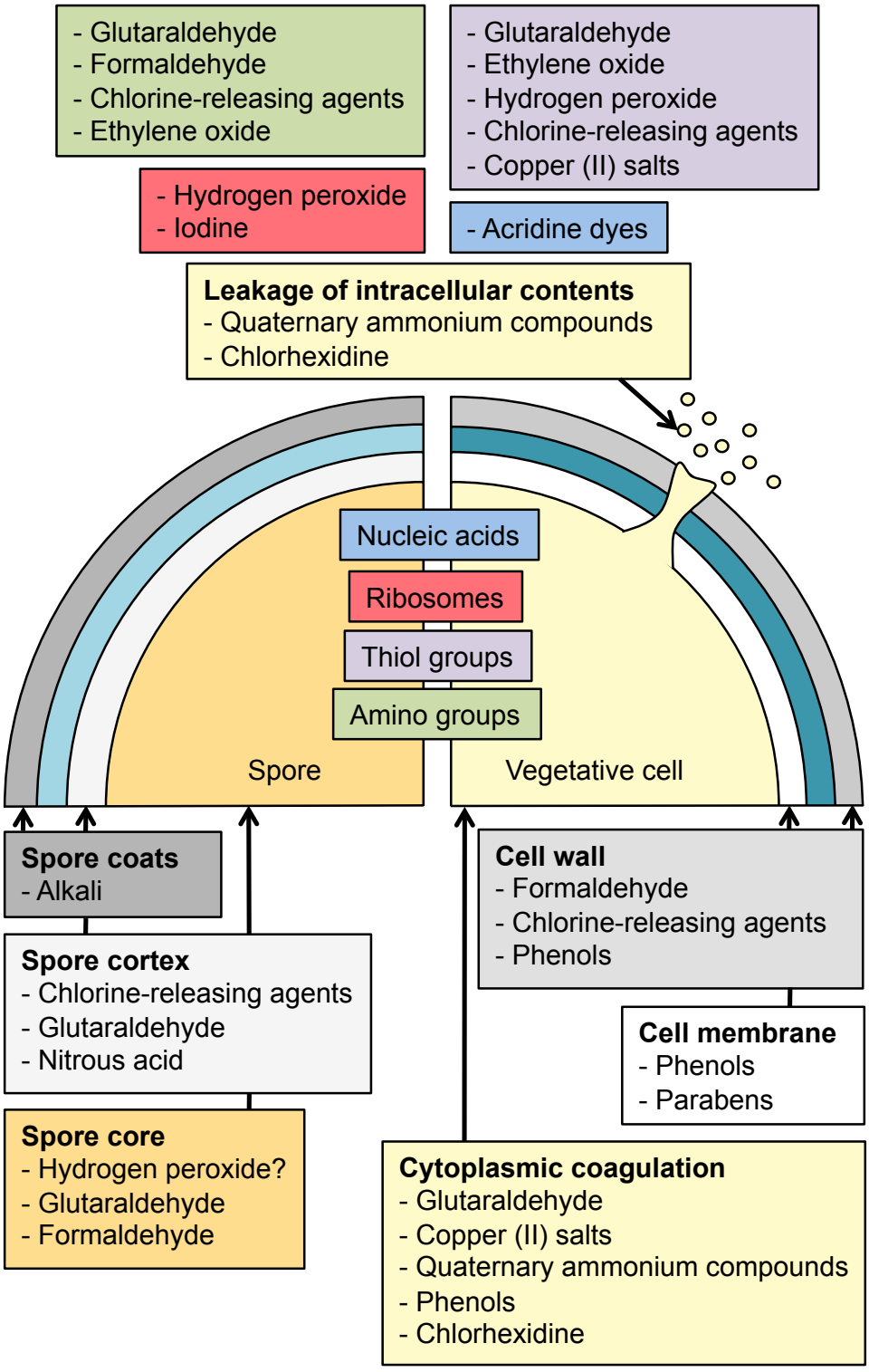
Currently in the UK, chlorine-containing agents that release 1000 ppm of chlorine are recommended for cleaning areas occupied by CDI patients either with a detergent pre-clean or in combination with a detergent (Department of Health, 2009); chlorine-based agents have a broad spectrum of activity (Fig. 4.1). However, no such recommendation has been made for the disinfection of laboratory surfaces that potentially get contaminated with high concentrations of *C. difficile* vegetative cells and spores.

4.1.9. Differences in susceptibilities

Spores are resistant to several disinfectants as mentioned above, especially in the presence of organic matter (Fawley *et al.*, 2007; Wheeldon *et al.*, 2008b). Vegetative cells have a lower ability to survive in air and are more susceptible to the effect of sporicides (Jump *et al.*, 2007; Wheeldon *et al.*, 2008c). But the key question is whether there is any difference in the susceptibilities of different strains of *C. difficile* to different agents.

Differences in the sporulation capacities of different ribotypes of *C. difficile* have been reported. Studies have shown that the hypervirulent ribotype 027 is capable of robust spore production (Akerlund *et al.*, 2008; Merrigan *et al.*, 2010) and the epidemic ribotype 001 produces significantly high numbers of spores compared to non-prevalent strains. The sporulation of ribotype 001 was found to be further enhanced when exposed to non-chlorine-based disinfectants (Wilcox & Fawley, 2000) which could explain its extensive dissemination and persistence in the environment. In the study by Wullt and co-workers, no differences in susceptibilities of different strains were observed to either glutaraldehdye, peracetic acid or acidified nitrite (Wullt *et al.*, 2003b); strains belonging to serogroups A and C were used along with a common nosocomial strain. Similarly, during the testing of a peroxy compound, it was found that the spores of ribotype 027 were inactivated as efficiently as those of the other strains tested (Horejsh & Kampf, 2010). However, whether other agents can have similar effects on both vegetative cells and spores needs to be explored.

Fig. 4.1. Mechanisms of inactivation by some biocides



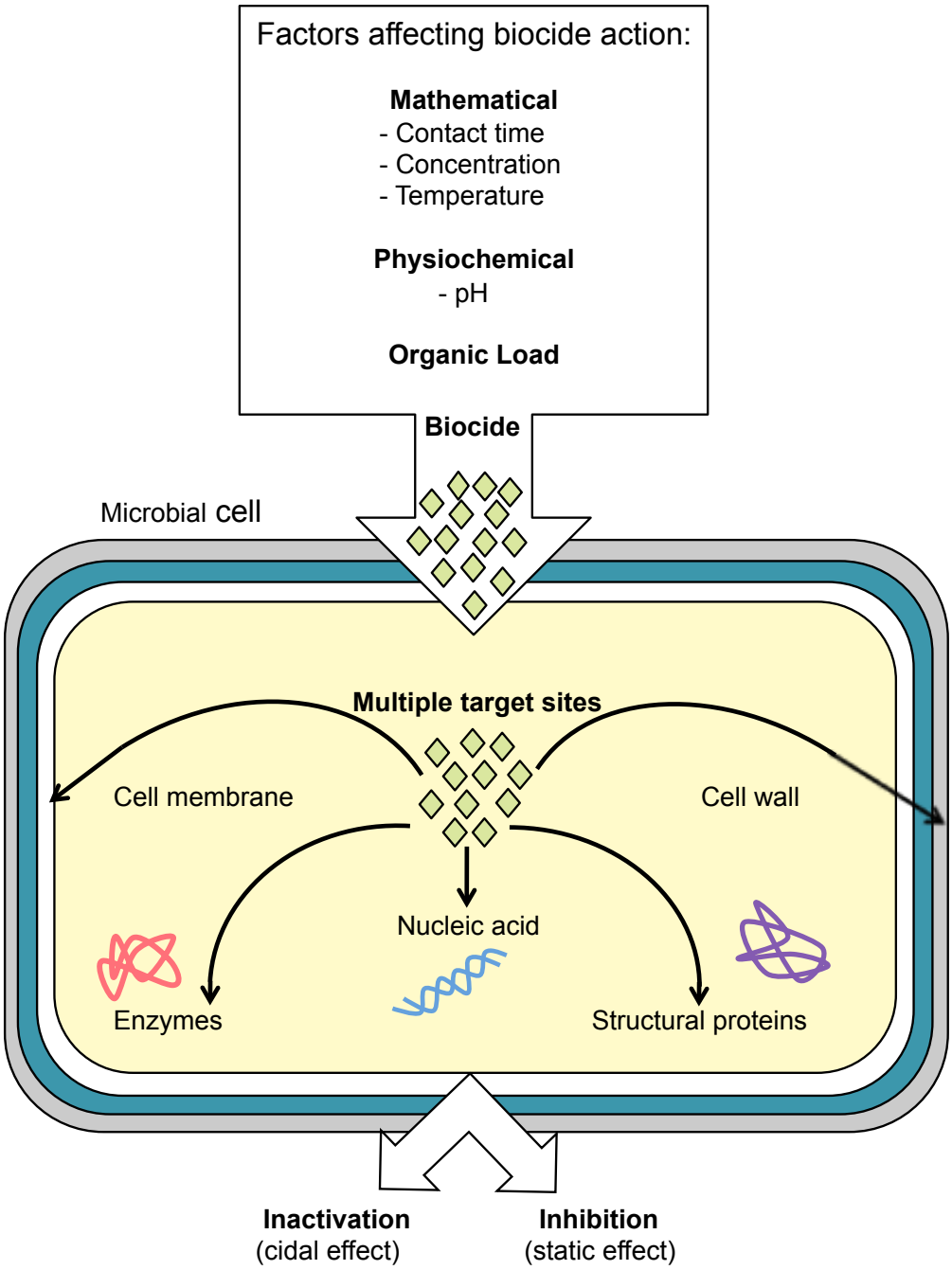
Chlorine-releasing agents, hydrogen peroxide and glutaraldehdye have a broad spectrum of activity against vegetative cells and spores of bacteria, which makes them some of the most frequently used disinfectants. Adapted from Russell *et al.*, 1997.

4.1.10. Static or cidal?

Several types of agents exist to decontaminate the environment; clear definitions of the different types of agents (Block, 2001) help us to identify the agent most suitable to achieve the desired outcome. A disinfectant is a chemical or physical agent which is able to eliminate undesired microorganisms from the inanimate environment, but may not kill bacterial spores. A disinfectant can sometimes be referred to as a germicide or a bactericide; however, a germicide generally refers to an agent that is able to kill microorganisms that are not bacteria, like fungi and viruses. A sporicide is an agent which destroys bacterial spores. In strict terms, a disinfectant can bring about decontamination of an environment, that is, it can render an item or surface free of pathogens to such an extent that it is unable to transmit pathogens and is safe to handle. On the other hand, a sporicide can be considered to be a sterilant as it is able to render an area free of the more resistant forms of microorganisms. Some agents can be bacteriostatic, that is, they can prevent the growth of bacteria and other living forms, but not destroy them. The difference between cidal and static activity of agents can sometimes depend on factors like temperature, time and pH (Fig. 4.2). For many agents, the outcome is dependent on cleaning (or pre-cleaning) with detergent, which involves the removal of visible dirt like soil, blood, proteins and debris from surfaces, before decontamination.

With respect to *C. difficile*, selected disinfectants are required to be bactericidal as well as sporicidal. Many commonly-used bactericidal agents like phenols, quaternary ammonium compounds, biguanides and alcohols are sporistatic; they may prevent the germination or outgrowth of spores but are sporicidal only at elevated temperatures (Russell, 1990; Russell, 1998). Even sporicidal agents such as aldehydes, chlorine compounds, peroxy acids and hydrogen peroxide are sporicidal at only very high concentrations (Russell, 1990). Although bacteriostatic and sporistatic concentrations for an individual chemical disinfectant might be similar, sporicidal concentrations are often considerably higher than those that are bactericidal (Russell, 1998). These characteristics of disinfectants can be determined by a range of standardised tests.

Fig. 4.2. Microbial inactivation by disinfectants: factors affecting it and possible outcomes



Biocides or disinfectants, unlike antibiotics, have a variety of target sites in bacterial cells; they can exert non-selective primary and secondary toxic effects on target cells. The efficacy of a biocide can depend on its concentration, the duration of exposure, environmental factors such as temperature, pH and organic matter and most importantly, the bacterial load. Adapted from Russell *et al.*, 1997.

4.1.11. Disinfectant testing

Over the years, a variety of methodologies have been developed to test the efficacy of disinfectants internationally (Reybrouck, 2007; Russell, 1998; van Klingeren, 2007). There are three stages in disinfectant testing as described by the European Committee for Standardisation (CEN) guidelines, CEN/TC 216 (Humphreys, 2010; Reybrouck, 2007). The first phase involves laboratory tests like suspension tests to determine the basic concentration-time dynamics of the disinfectant against the selected bacteria. In the second stage, these suspension tests can be modified to incorporate factors like organic matter, making the results more relevant to a practical situation. Other laboratory tests mimicking real-life conditions are also performed to evaluate activity under the actual conditions of use. In the third stage, field tests are performed, but these are generally more difficult to perform and analyse due to the variation in conditions between one location and another.

When the decontamination of spore-forming bacteria like *C. difficile* is required, it is essential to determine the sporicidal activity of disinfectants. As part of preliminary testing, bacteriostatic concentrations of disinfectants can be determined by broth- and agar-dilution techniques; the minimum inhibitory concentration (MIC) value thus obtained can be used to estimate the sporistatic concentration of the agent as they are generally closely related concentrations (Russell, 1998).

The tests to determine the sporicidal activity of disinfectants can be divided broadly into three categories: suspension tests, carrier tests and 'in-use' surface tests. In a suspension test, a standardised inoculum of bacteria is added to a defined volume of disinfectant and after different times of testing, an aliquot of this mixture is removed and cultured to check for growth. Interfering substances like organic matter and blood can be introduced into these tests. Good mixing of test bacteria and agents in suspension tests improves their reproducibility (Humphreys, 2010). However, there may be a carry-over of small amounts of the disinfectant into the recovery medium that might give a false result; a bacteriostatic effect could be interpreted as a bactericidal effect (Shippen, 1928). The Association Française de Normalisation

(AFNOR) suspension test is one such method to determine sporicidal activity of disinfectants (Espigares *et al.*, 2003; Herńandez *et al.*, 2000; Russell, 1998).

Carrier tests involve the use of inanimate objects like steel, porcelain and even silk thread contaminated with a standardised inoculum. Once the contaminant has dried on the carrier, it is submersed in the disinfectant for a defined period of time, followed by neutralisation of the agent and transfer of the carrier to growth medium. The disadvantage of this type of test is that it is difficult to standardise the number of bacteria dried onto a surface and also to analyse the loss of viable cells due to drying on the surface (Reybrouck, 2007; van Klingeren, 1995). The Association of Official Analytical Chemists (AOAC) sporicidal test is an example of a widely used carrier test as described in several studies (Gröschel, 1991; Rutala *et al.*, 1993b).

Surface tests are performed to get a more real understanding of the interaction between the disinfectant and contaminating bacterium under practical conditions. A standardised inoculum is dried onto a surface like stainless steel or polyvinyl chloride. Subsequently, a small volume of disinfectant is added to the surface for a specified contact period. The viable organisms remaining on the surface are recovered and enumerated. These tests are more likely to reflect a real-life situation as the ratio of disinfectant to inoculum is decreased (Humphreys, 2010; van Klingeren, 2007). Such tests are described in documents such as BS EN 14349 and BS EN 13697 of the British Standards Institute (Humphreys, 2010).

While performing such sporicidal assays, the individual steps must be standardised to get reliable and reproducible results. The method for preparing the spore suspensions must be standardised and the inocula must be the same in every experiment (Russell, 1998). The medium in which the spores are prepared could affect the action of the disinfectants and must be carefully evaluated (Perez *et al.*, 2005). Further, the disinfectant must be adequately neutralised after the desired time of exposure to prevent sporistasis in the recovery medium (Russell, 1998). This can be achieved by diluting the disinfectant to sub-inhibitory levels, chemical neutralisation with a non-toxic agent or by washing the filter used to remove the agent, followed by incubation

of the filter on solid media. Also, it is essential to ensure that conditions for the revival of damaged spores are suitable in order to avoid false negative results; this might require prolonged incubation periods or alteration of the recovery medium. The aim of this study was to identify the most suitable laboratory agents for the decontamination of surfaces contaminated with *C. difficile*. The agents used for testing were thus selected from those available within our laboratory. They included Decon 90, Microsol 3+, TriGene Advance and Virkon, as described in Table 2.4. For comparison with decontamination that is routinely performed in hospitals, Actichlor, was also included in this study. The strains used for all the studies were the reference strains, 630 and VPI 10463 and the epidemic ribotypes 027, 001 and 106.

4.2. Methods

The first stage in disinfectant testing involved qualitative tests; minimum inhibitory concentration (MIC) testing was performed using vegetative cells of the five *C. difficile* strains by the Wadsworth agar-dilution method and by broth-microdilution (2.8.2). MIC testing by agar-dilution was also performed for 23 clinical isolates (Table 2.2). For *C. difficile* spores, survival assays were performed (2.8.4). Further, quantitative assays were performed to determine the extent of sporicidal activity by the measuring the $\log_{10}$ reduction (2.8.5) in suspension tests. The scope of these tests was extended to clean and dirty conditions - the absence and presence of organic matter, respectively - to understand how environmental conditions affect decontamination. Also, a practical test (2.8.6) was performed to determine the efficacy of the decontamination of surfaces contaminated with *C. difficile* spores. The surfaces selected were those commonly encountered in laboratories as well as hospitals: aluminium, glass, plastic, vinyl tiles and ceramic tiles. Further, the effect of sub-MIC concentrations of the agents on the sporulation capacity of the different *C. difficile* strains was tested (2.8.7). Finally, to understand the extent to which *C. difficile* can disseminate in and around a laboratory performing *C. difficile* research, environmental sampling was performed using contact plates (2.9.1). The colonies were enumerated from the plates and ribotyping was performed (2.9.2).

4.3. Results

Through a series of quantitative and qualitative tests it was possible to determine which of the commonly available disinfectants in our laboratory were suitable to decontaminate our immediate environment of *C. difficile*. Also, the level of *C. difficile* contamination within our laboratory was examined.

4.3.1. MIC determination

Growth of vegetative cells of the five *C. difficile* strains was effectively suppressed by all the agents tested. The MICs of the agents for all strains were lower than the manufacturer's recommended working concentration as determined by agar-dilution and are represented as a fraction of the recommended working concentration in Table 4.1. The same results were obtained by broth-microdilution. However, for Actichlor, Decon 90 and Virkon, higher concentrations were required to destroy vegetative cells of the epidemic ribotypes 027 and 001. Microsol 3+ appeared to be the most effective agent, active at a 128-fold dilution of the recommended concentration, followed by TriGene Advance, which was effective at a 32-fold dilution of the recommended concentration. Disinfectant sensitivity profiles of the clinical isolates of all ribotypes, except ribotype 001, matched the profile of ribotype 106.

Table 4.1. Minimum inhibitory concentration (MIC) of the agents for vegetative cells of five *C. difficile* strains determined by agar-dilution and broth-microdilution and represented as a fraction of the recommended working concentration

Agent	Minimum Inhibitory Concentration				
	Strain 630	Strain VPI 10463	Ribotype 027	Ribotype 001	Ribotype 106
Actichlor	1/8	1/8	1/2	1/8	1/8
Decon 90	1/4	1/4	1/2	1/2	1/4
Microsol 3+	1/128	1/128	1/128	1/128	1/128
TriGene Advance	1/32	1/32	1/32	1/32	1/32
Virkon	1/8	1/8	1/4	1/4	1/8

4.3.2. Effect on spore viability

The agents tested were also found to be sporicidal at the recommended concentration in suspension tests at 2 min, 10 min and 30 min of testing (Table 4.2). Once again it was observed that ribotype 027 required a greater concentration of Actichlor for destruction of its spores; spores survived at a 2-fold dilution of the recommended concentration. This was also true for the epidemic ribotype 106. For Virkon, any dilution below the recommended concentration was not sporicidal at 2 min, making it a less effective disinfectant than Actichlor. These studies demonstrate the importance of accurate preparation of disinfectants before use. Interestingly, Microsol 3+ and TriGene Advance were found to be sporicidal at a 5-fold dilution of the recommended concentration even at 2 min of exposure. However, this could be a result of prolonged exposure to low concentrations of the active ingredients in these agents as no neutraliser or washing step was included.

Table 4.2. Minimum sporicidal concentration of five agents represented as a fraction of the recommended working concentration after different times of exposure

Agent [a]	Time of exposure	Minimum Sporicidal Concentration				
		Strain 630	Strain VPI 10463	Ribotype 027	Ribotype 001	Ribotype 106
Actichlor	2 min	<1/5	<1/5	1/2	<1/5	1/2
	10 min	<1/5	<1/5	<1/5	<1/5	<1/5
	30 min	1/5	<1/5	<1/5	<1/5	<1/5
Decon 90	2 min	1	1/2	1/2	1	1/2
	10 min	1/2	<1/5	<1/5	1/2	<1/5
	30 min	<1/5	<1/5	<1/5	<1/5	<1/5
Virkon	2 min	1	1	1	1	1
	10 min	1	<1/5	1	<1/5	<1/5
	30 min	1	<1/5	1	<1/5	<1/5

[a] The minimum sporicidal concentration for Microsol 3+ and TriGene Advance for all the strains was less than 1/5 recommended concentration at 2, 10 and 30 min.

4.3.3. Determination of log_{10} reduction

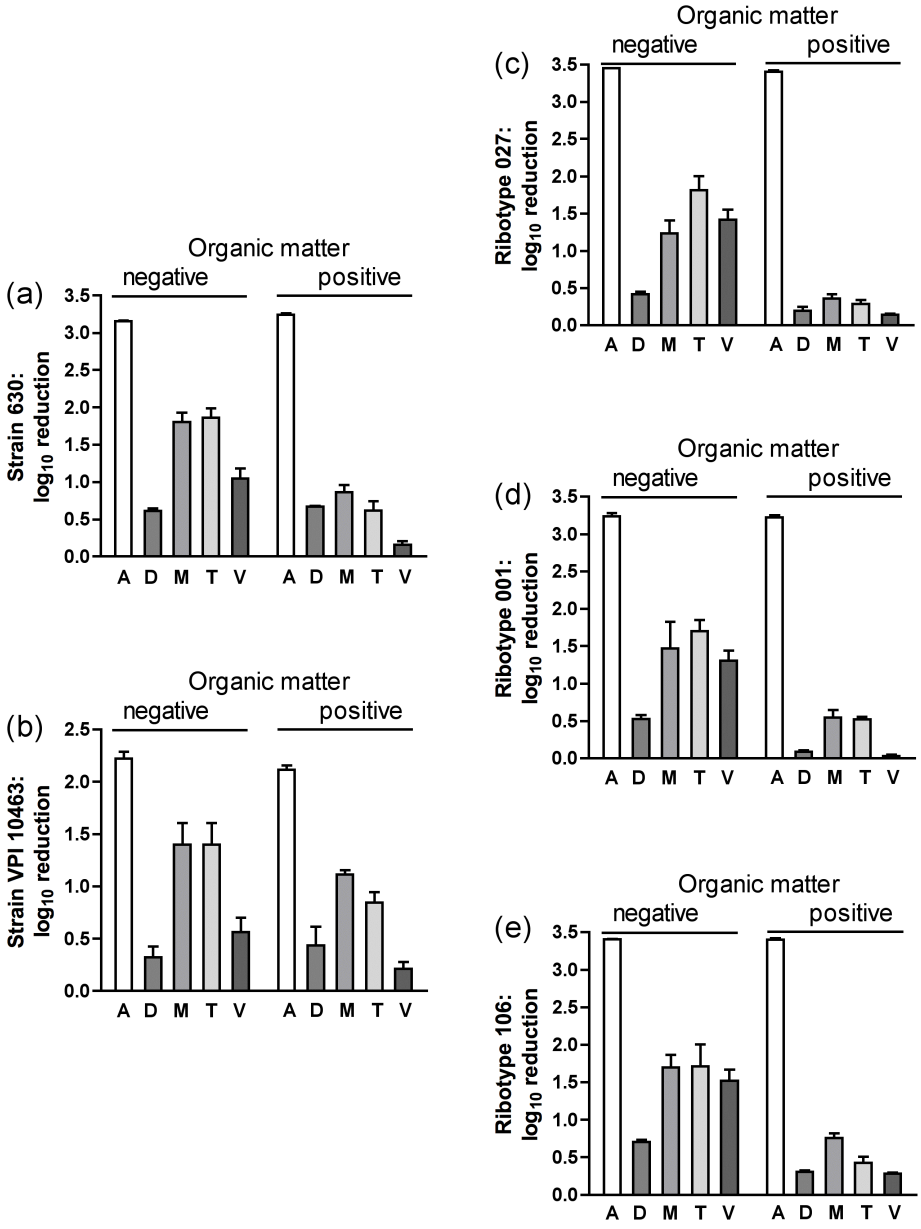
All the selected agents, except Decon 90, are marketed as sporicidal agents. Thus, it was of interest to determine the log_{10} reduction in spore numbers brought about by them at the recommended concentration. To avoid exposure of the spores to small traces of any active ingredients, they were washed thoroughly after being treated with the agents. Under clean conditions in the absence of organic matter, Actichlor was found to be the most effective sporicidal agent, bringing about a 3 log_{10} reduction of *C. difficile* spores at 10 min, while Microsol 3+, TriGene Advance and Virkon only caused an approximately 1.5, 1.7 and 1.2 log_{10} reduction, respectively (Table 4.3). In the presence of organic matter, the efficacy of all the cleaning agents, except Actichlor, dropped considerably. The drop in efficacy of Virkon from approximately 1.2 log_{10} in the absence of organic matter to approximately 0.2 log_{10} in its presence was especially notable. Also, interestingly, in the presence of organic matter, for all the agents except Actichlor, the log_{10} reduction for the epidemic strains was more markedly perceptible as compared to the non-epidemic strains (Fig. 4.3). As these experiments were not performed with more than 10^3 spores/test, it is possible that the activity of Actichlor has been underestimated and it brings about a greater log_{10} reduction at the time tested.

Table 4.3. Average log_{10} reduction in spores of five *C. difficile* strains in the absence and presence of organic matter

Agent	log_{10} reduction (Mean ± Std. Error) [b]	
	Organic matter negative	Organic matter positive
Actichlor	3.093 ± 0.2239	3.076 ± 0.2429
Decon 90	0.5159 ± 0.06722	0.3384 ± 0.101
Microsol 3+	1.519 ± 0.1025	0.7288 ± 0.1294
TriGene Advance	1.698 ± 0.0806	0.5399 ± 0.09384
Virkon	1.171 ± 0.1705	0.1657 ± 0.04113

[b] The log_{10} reduction is an average of the five *C. difficile* strains

Fig. 4.3. Efficacy of five agents (log_{10} reduction) against five strains of *C. difficile* in the absence and presence of organic matter



The efficacy of Actichlor (A), Decon 90 (D), Microsol 3+ (M), TriGene Advance (T) and Virkon (V) was tested against the spores of five strains of *C. difficile*. Only Actichlor effectively destroyed spores of all the strains in the absence or presence of organic matter, causing a 3 log_{10} reduction in spores. The efficacy of all the other agents decreased significantly in the presence of organic matter, especially in terms of destroying spores of the epidemic ribotypes 027 (c), 001 (d) and 106 (e). Bars indicate +/- SEM of 2 experiments.

4.3.4. Decontamination of surfaces

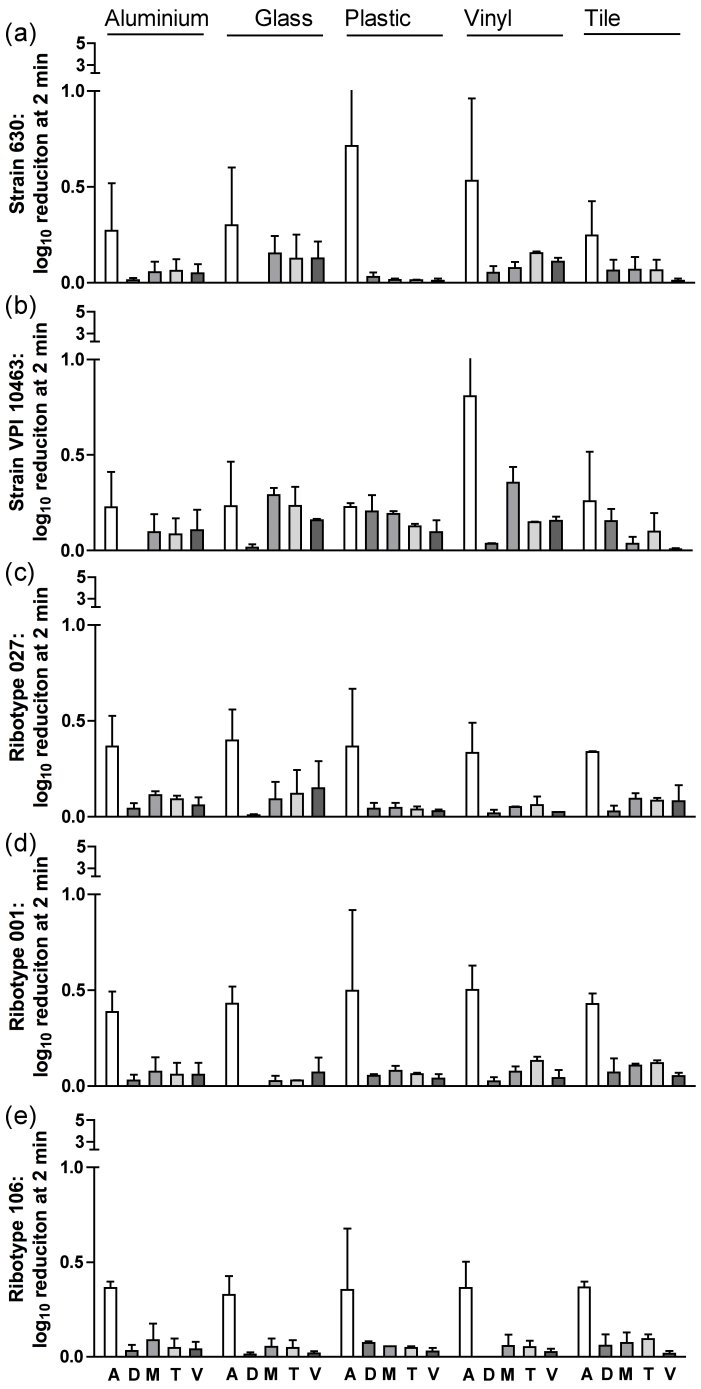
To test the decontamination of surfaces using the selected agents, 1 cm^2 areas were artificially contaminated with 10^3 spores of each strain, allowed to dry and then cleaned, in an attempt to mimic a real-life situation. Further, the surface was washed by aspiration of disinfectant and subsequent washes. It was observed that only chlorine-releasing Actichlor was able to completely decontaminate all the surfaces tested; however, more than 2 min (Fig. 4.4.a) and up to 10 min (Fig. 4.4.b) were required to see this effect. For all the other agents, although there was a greater log_{10} reduction in spore numbers after 10 min of treatment as compared to 2 min, it was still negligible compared to the initial level of contamination. Here too it was found that the spores of the epidemic strains were less effectively destroyed by all the non-chlorine agents. The spores of strain 630, which was previously epidemic in Scotland, were also more resistant to cleaning, while spores of VPI 10463, which is rarely isolated from patients, were the most effectively destroyed.

4.3.5. Effect of sub-MIC concentrations on sporulation

The five *C. difficile* strains were cultured for 5 d in the presence of concentrations corresponding to 1/4 of the MIC of all the agents, except Actichlor. The growth of all the strains as measured by OD_{600} was considerably lower only in the presence of TriGene Advance (Fig. 4.5.a). The presence of sodium taurocholate in the medium did not affect the growth of the strains. However, it did augment sporulation in all the strains when grown with Decon 90 and Microsol 3+.

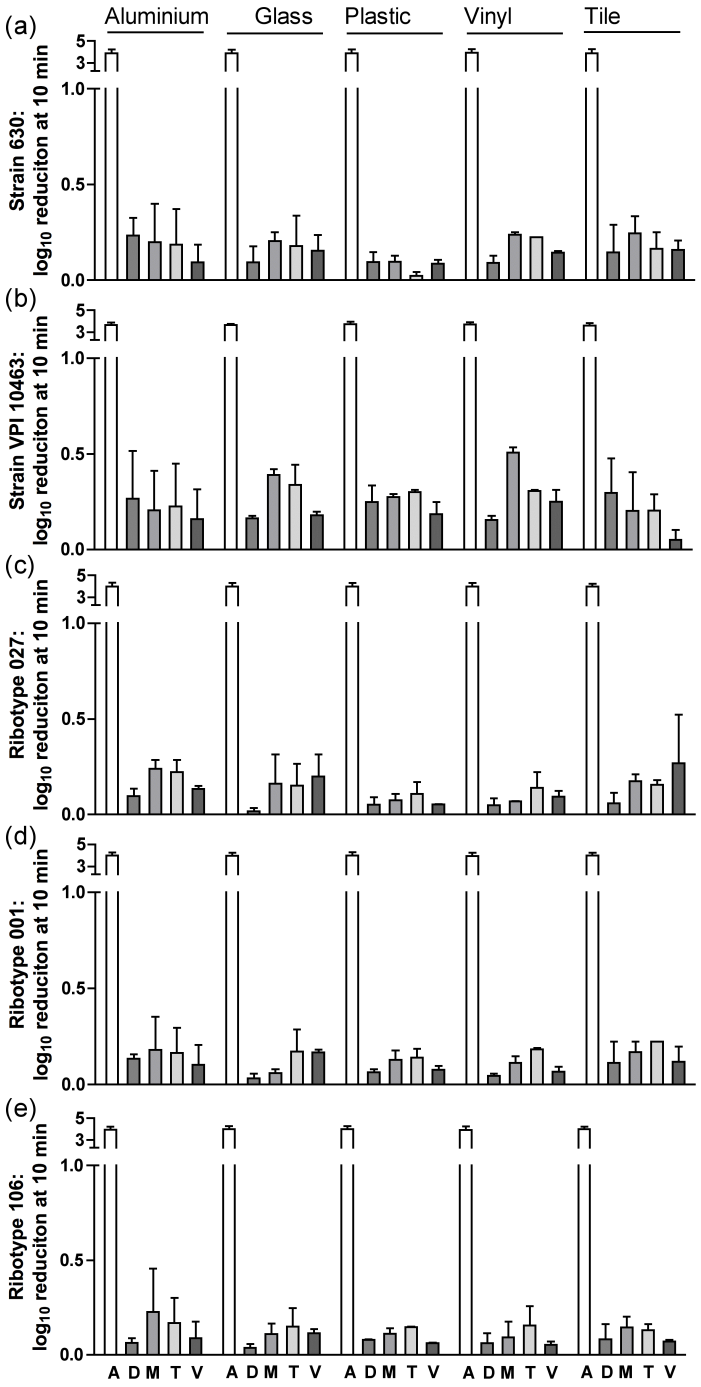
Exposure to sub-MIC concentrations of the agents led to high numbers of spores being produced by all the epidemic strains; this increase was most notable in ribotype 001, followed by ribotypes 027 and 106, respectively (Fig. 4.5.b). Overall, the epidemic strains appeared to be more affected by exposure to sub-MIC concentrations of different agents as compared to the reference strains. Also, the spores produced by ribotype 001 appeared to be more resilient when compared to ribotypes 027 and 106 as the presence of taurocholate had a lower impact on their germination and outgrowth.

Fig. 4.4.a. Level of surface decontamination after 2 minutes of exposure to five agents



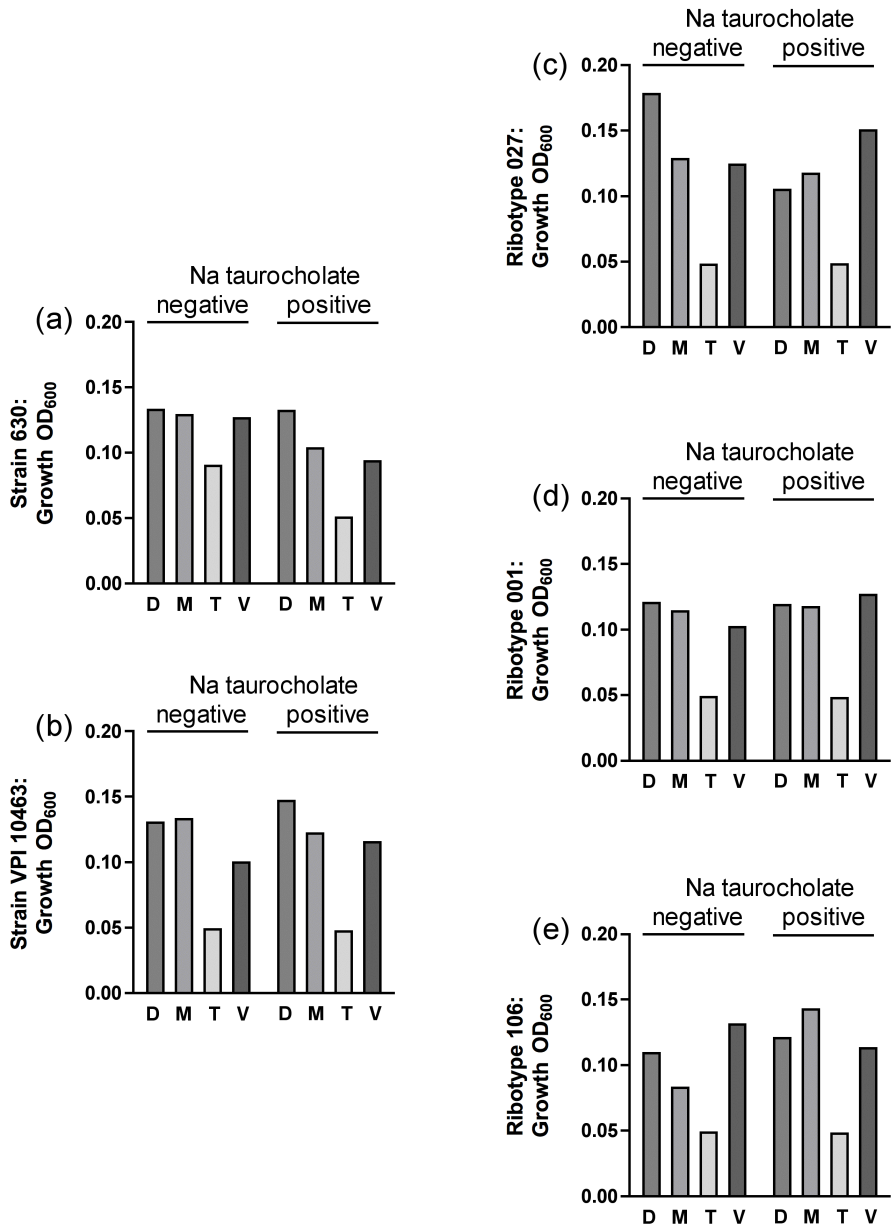
After 2 min of exposure, Actichlor (A) was found to be the most effective decontaminant, while Decon 90 (D), Microsol 3+ (M), TriGene Advance (T) and Virkon (V), fared poorly. However, complete decontamination of the surfaces was not achieved even with Actichlor, illustrating the need for longer exposure of contaminated surfaces to cleaning agents in order to effectively destroy spores of *C. difficile*. Bars indicate +/- SEM of 2 experiments.

Fig. 4.4.b. Level of surface decontamination after 10 minutes of exposure to five agents



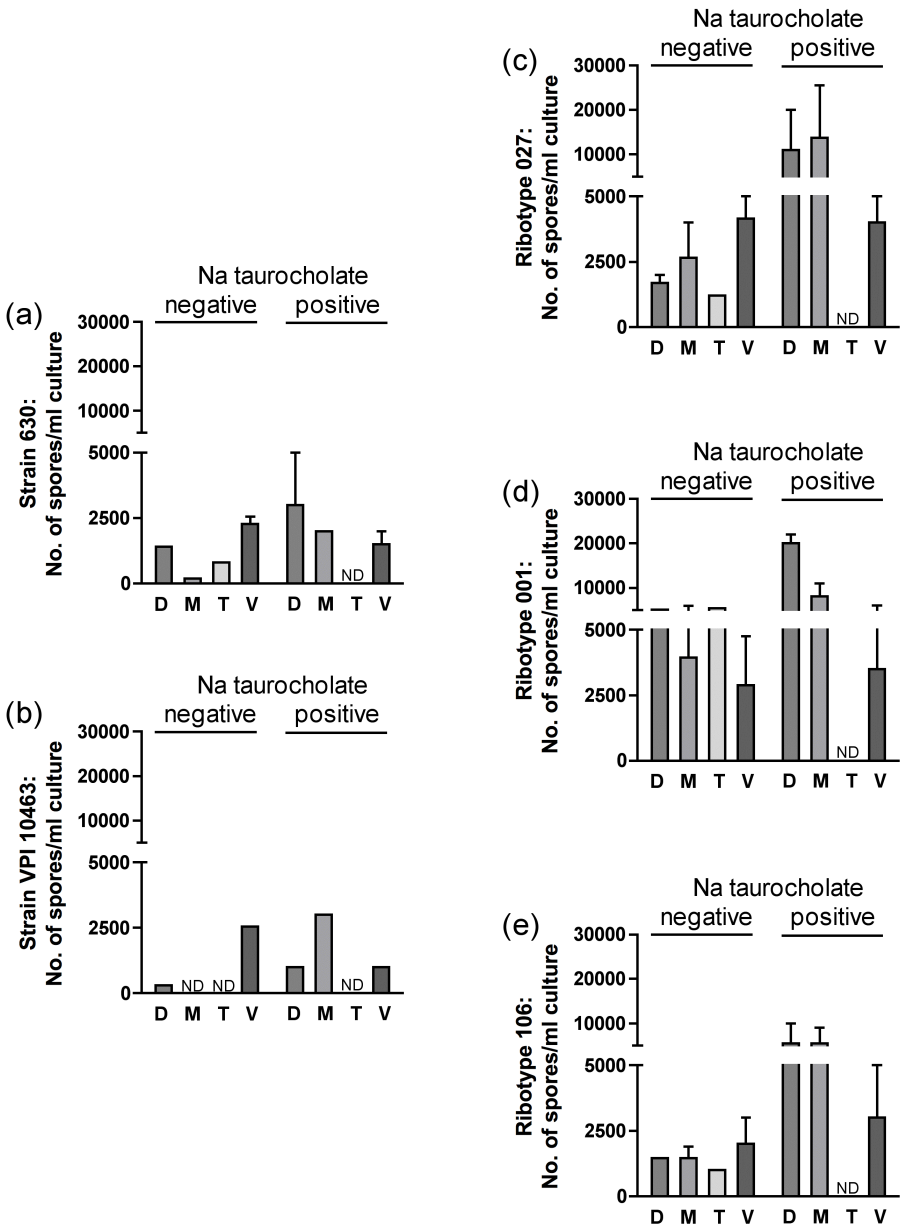
After 10 min of exposure to the agents, only Actichlor (A) successfully destroyed all *C. difficile* spores. The other agents brought about a negligible log_{10} reduction, especially against the epidemic ribotypes 027 (c), 001 (d) and 106 (e). Bars indicate +/- SEM of 2 experiments.

Fig. 4.5.a. Effect of sub-MIC concentration of non-chlorine agents on growth of five *C. difficile* strains



When grown anaerobically in the presence of sub-MIC concentrations of all the agents except Actichlor, the growth of the five *C. difficile* strains was only markedly affected by TriGene Advance (T), suggesting that it might be bacteriostatic at the concentration used. There was no notable difference between the growth of the strains in the presence of Decon 90 (D), Microsol 3+ (M) or Virkon (V). The presence of sodium taurocholate in the medium did not affect the growth of the strains.

Fig. 4.5.b. Effect of sub-MIC concentration of non-chlorine agents on sporulation of five *C. difficile* strains



Exposure to sub-MIC concentrations of non-chlorine-based agents appeared to enhance the sporulation of *C. difficile* ribotype 001 (d) considerably, followed by ribotype 027 (c) and 106 (d), respectively. The presence of sodium taurocholate in the medium aided sporulation at sub-MIC levels of Decon 90 (D) and Microsol 3+ (M), but spores were not detected (ND) at sub-MIC levels of TriGene Advance (T) under the same conditions. Bars indicate +/- SEM of 2 experiments.

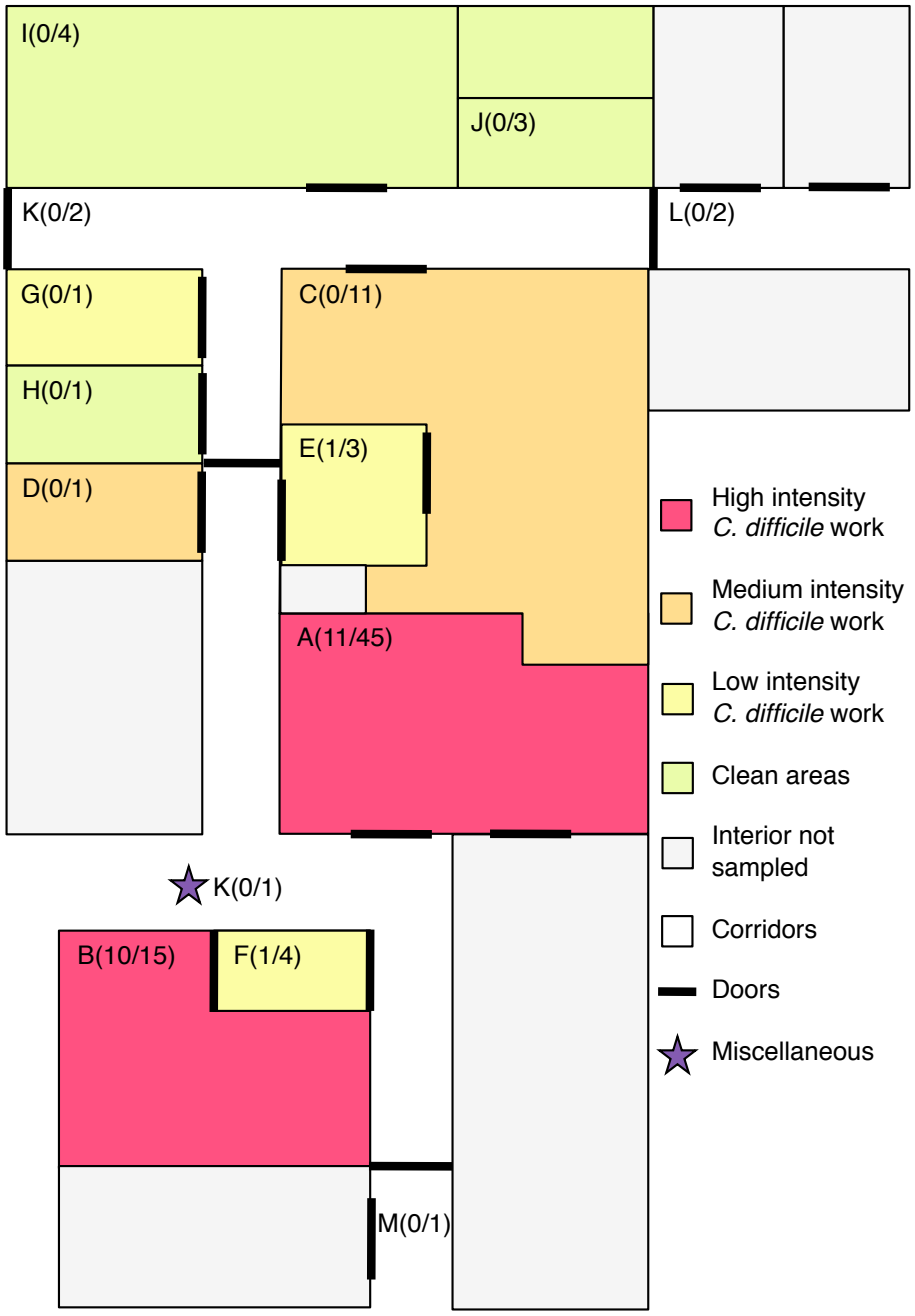
4.3.6. Environmental sampling

To examine the level of contamination by *C. difficile* in and around our laboratory, 93 sites were selected for sampling (Fig. 4.6. and Table 4.4). Sampling was performed in duplicate using contact plates with either CCEY agar or blood agar. Samples were collected from different areas in the laboratory carrying out varying degrees of work with *C. difficile*. Also, areas around the laboratory were sampled, including clean media preparation areas, corridors and offices.

Of the 93 sites sampled, 23 (24.73%) were found to be positive for *C. difficile*. As expected, majority of the positive samples were detected in the areas of highest intensity of *C. difficile* work; 11 of the 45 sites in area A and 10 of the 15 sites in area B were positive. Interestingly, the other two positive sites were in areas E and F, showing the potential for spread even in areas that should be mainly *C. difficile*-free. Along with *C. difficile*, a large number of other anaerobes were isolated from all the sites (Fig. 4.7).

A total of 60 *C. difficile* colonies were isolated from all the positive sites. Ribotyping was performed on these to identify if the contamination of the environment was due to a single strain or several different types. The typing was not successful for samples from three of the 23 sites. Of the 60 colonies, PCR reactions for only 38 (63.33%) were successful. Although the gel image was not of suitable quality to analyse using the ribotyping software, four distinct banding patterns were obtained (Fig. 5.6.). The types were assigned as i, ii, iii and iv, which constituted 57.89% (22/38), 21.05% (8/38), 2.63% (1/38) and 18.42% (7/38), respectively of the total number of typed colonies. Colonies of single types were obtained from 17 sites and three sites yielded two colony types (Fig. 4.8). These sites were in areas A, B and E and were a bench, the interchange tray of an anaerobic chamber and the keypad of a centrifuge, respectively. The most predominant type i strain was one of the two *C. difficile*-types identified from each of these sites.

Fig. 4.6. Map of the areas selected for environmental sampling in and around the laboratory



Ninety-three sites in and around our laboratory were sampled for *C. difficile* using contact plates. The areas have been colour-coded according to the intensity of work with *C. difficile* being carried out in them and they have been labeled A to M. The number of positive samples detected/the total number of sites sampled are stated in the parentheses alongside each area label. As expected, the areas in which maximum *C. difficile* work is performed were found to be the most contaminated. The specific sites sampled in each area are listed in Table 5.4.

Table 4.4. List of the 93 sites sampled in each area, of which the 23 encased in boxes were found to be *C. difficile*-positive

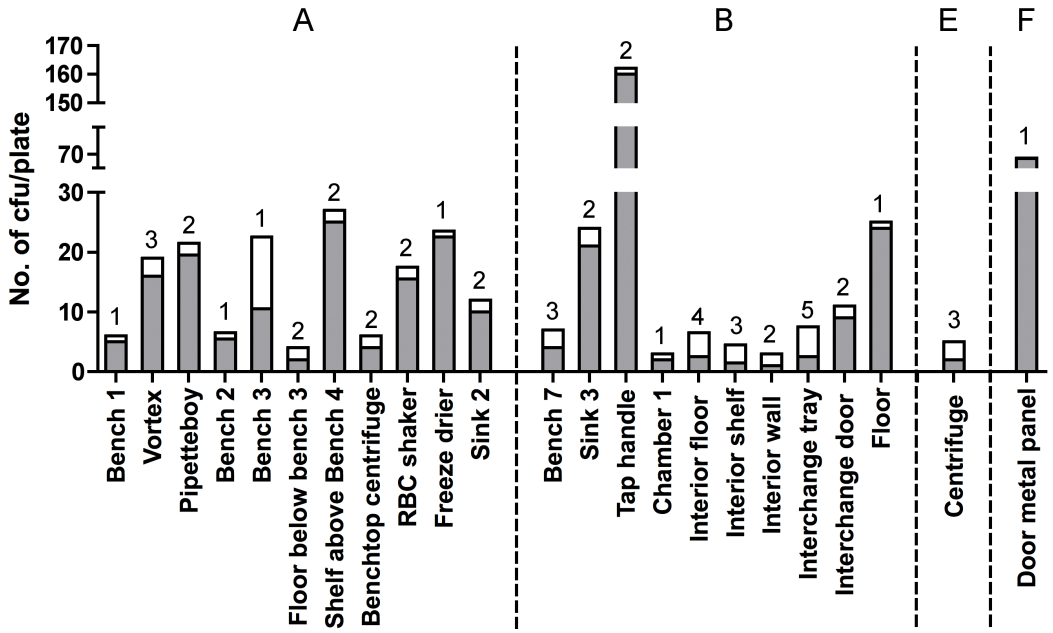
A: 45 sites	Sink 2 (outer edge)	Non-*C. diff* worker's gloves (palm)
Bench 1 and on it:	Door of adjacent culture lab	Non-*C. diff* worker's gloves (top)
Discard jar (lid)	37°C incubator door	D: 1 site
Disinfectant spray bottle (base)	37°C incubator (interior wall)	Waste disposal area
Vortex (upper surface)	Fridge (handle)	E: 3 sites
Pipetteboy (handle)	Fridge (interior door)	Centrifuge (keypad)
Pipette rack (upper suface)	Storage cupboard (door)	Plate drier (door)
Pipette-tip box (lid)	Computer keyboard	Scales (surrounding area)
Multichannel pipette (base)	Ceiling	F: 4 sites
Lab book of user (cover)	Air vent on ceiling	Door (exterior laminate surface)
Top drawer below bench	B: 15 sites	Door (handle)
Back of chair	Bench 7	Door metal panel (outwards)
Base of chair	Sink 3 (interior) and:	Door metal panel (inwards)
Floor under it	Tap handle	G: 1 site
Lab coat of user of bench 1	Wall above it	Bead beater (lid)
Fingers of user of bench 1	Anaerobic chamber 1 (door)	H: 1 site
Bench 2 and on it:	Of anaerobic chamber 2:	Media preparation area
Wire gauze (upper surface)	Interior floor	I: 4 sites
Bench 3 and:	Interior shelf	Office desk 1 and:
Metal box on shelf above it	Interior wall	Phone on it (receiver)
Floor below it	Interchange tray	Office desk 2
Bench 4	Interchange door	Carpet (at the entrance)
Shelf above bench 4	Floor below it	J: 3 sites
On bench 5: Bijoux bottle	Music player	Counter
Pipette rack	Fridge/freezer (door)	Microwave (door)
Benchtop centrifuge (lid)	-70°C freezer (door)	Coffee table
PCR machine (lid)	C: 11 sites	K: 2 sites
RBC shaker (vertical surface)	Bench 8	Exit button
Shaker (upper surface)	Bench 9	Floor
Shelf above bench 6 and:	Desk	L: 2 sites
Freeze drier on it	QPCR machine (door)	Door of ladies' toilet
Sink 1 (interior) and:	Spectrophotometer (top)	Door of mens' toilet
Tap handle	Power pack (upper surface)	M: 1 site
Soap dispenser	Heating block (upper	Door handle on the cold room door
Wall above it	-20°C freezer (door)	Miscellaneous:
Floor below it	Computer mouse	Sole of a lab worker's shoe

Table 4.5. *C. difficile*-positive sites and numbers and types of *C. difficile* colonies identified from each of them

Site	Total no. of *C. difficile* colonies	No. of typed colonies of each assigned type			
		i	ii	iii	iv
Bench 1	1	1			
Vortex	3		2		
Pipetteboy	2	2			
Bench 2	1		1		
Bench 3	12	3	1		
Floor below bench 3	2	2			
Shelf above bench 4	2	2			
Benchtop centrifuge	2	2			
RBC shaker	2	1			
Freeze drier	1				1
Sink 2	2				2
Bench 7	3	3			
Sink 3	3		2		
Tap handle	2		1		
Chamber 1	1	**	**	**	**
Interior floor	4	**	**	**	**
Interior shelf	3	**	**	**	**
Interior wall	2				1
Interchange tray	5	4			1
Interchange door	2				1
Floor	1			1	
Centrifuge	3	2	1		
Door metal panel	1				1

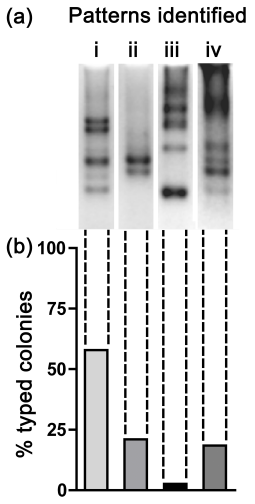
** Undetermined

Fig. 4.7. Sites contaminated with *C. difficile*



For each of the 23 *C. difficile*-positive sites, the total number of colonies obtained on non-selective blood agar (grey area) and the number of *C. difficile* colonies among them (white area) were enumerated. The specific number of *C. difficile* colonies isolated from each site are noted above the bars. Further, the positive sites are separated according to their location (A, B, E and F) as represented in Fig. 5.4.

Fig. 4.8. Types of *C. difficile* identified from the laboratory environment



PCR ribotyping was successfully performed on 63.33% of the colonies isolated from 20 of the 23 sites sampled. (a) Although the gel images were not of suitable quality to be analysed by the ribotyping software, on observing them by eye, 4 distinct banding patterns were identified among the samples. These were assigned as i, ii, iii and iv. (b) These assigned strain types i, ii, iii and iv constituted 57.89%, 21.05%, 2.63% and 18.42% respectively of the total number of typed colonies. From 17 sites, colonies of only one banding pattern were obtained; from 3 sites, two types of colonies were identified.

4.4. Discussion

'It would seem that tests of the sporicidal activity of disinfectants are, for practical purposes, best performed by pessimists' (Kelsey *et al.*, 1974). Although the results from the initial laboratory tests in this study were encouraging, when challenged with conditions normally encountered in the real-world, just one disinfectant stood out as a likely candidate for the battle against *C. difficile* contamination. Unfortunately, it is not one routinely used in the laboratory.

The primary challenge with disinfectant testing is that most of the accepted test methods do not mimic the real environment in a hospital or laboratory and the results thus do not translate to practical conditions; the requirement of a 3 $\log_{10}$ reduction under clean conditions in 60 minutes is clearly not realistic. In this study, the MIC results against vegetative cells indicated good efficacy of all the agents studied against the five *C. difficile* strains selected. Although exponential phase cultures consisting of mainly vegetative cells were selected for these tests, it is possible that there were some spores present. Using this method, it was not possible to identify if the agents had any cidal effects on either the vegetative cells or spores of *C. difficile*. It has previously been shown that minimum bactericidal concentrations (MBCs) can be much higher than inhibitory concentrations (Russell *et al.*, 1999). Thus, it is possible that for Decon 90, Virkon and even Actichlor, the manufacturers' recommended concentrations might not actually be cidal even for the vegetative cells of *C. difficile*; this is less likely for TriGene Advance and Microsol as their MICs are much lower than the recommended concentration.

In the next stage of testing too, with a standardised spore preparation, it appeared as though all the agents were sporicidal at the manufacturers' recommended concentrations even at 2 minutes of exposure. The method used ensured that a 10-fold dilution of the disinfectant concentrations was tested in the recovery medium. However, it is possible that yet again a sporistatic result was obtained due to the prolonged exposure of the spores to low but effective concentrations of the agents tested. Dilution of disinfectants in the recovery stage is generally considered to be

suitable (Russell, 1998) but as the effect of dilutions on spores was not validated independently, the killing of spores could not be confirmed. No growth was observed on the recovery agar from the dilutions that showed a static/cidal effect, suggesting that there was destruction of spores. However, it is clear that had a neutraliser been used or if the agents had been removed by washing, more conclusive results about the true effect of the agents would have been obtained. Of note from these results is the observation that for Decon 90, Virkon and Actichlor, the static/cidal effect was a result of using them at the recommended concentration or a 2-fold dilution of the same, which emphasises the importance of preparing these agents at the correct concentration. It was also interesting to find that higher concentrations of Actichlor, which is commonly used in hospitals, were required for inactivation of spores of ribotypes 027 and 106 in 2 minutes, suggesting the possibility that the epidemic ribotypes may have a greater resistance to commonly-used cleaning agents. The fact that no growth was observed for Microsol 3+ and TriGene, suggests that they have a strong cidal or static effect on *C. difficile*.

To confirm the efficacy of the agents, $\log_{10}$ reduction studies were performed in suspension tests and this time the agents were removed by thorough washing. Organic matter was also introduced into the tests to create practical conditions. From the results, it was clear that of the agents tested, Actichlor should be the agent of choice for the decontamination of *C. difficile*. The effect of all the other agents was mediocre even at 10 minutes in suspension and decreased further in the presence of organic matter. From these results, it could be confirmed that the results obtained in the previous set of experiments were due to sporistasis. Oddly, the generally observed decrease in efficacy of NaDCC disinfectants in the presence of organic matter (Ungurs *et al.*, 2011; Wheeldon *et al.*, 2008b) was not seen here with Actichlor. Also, there was no noteworthy difference in the effect of the biocides on the different *C. difficile* strains.

On a variety of clean non-porous surfaces too, only Actichlor was able to destroy all the spores seeded onto them but it required more than 2 minutes and up to 10

minutes for this result. These results for Actichlor are contrary to those of Ungurs and co-workers, who only observed a minimal reduction in *C. difficile* spores even after 120 minutes using 1000 ppm of free chlorine, as was used in this study (Ungurs *et al.*, 2011). In a real-life situation, 10 minutes of exposure to any agent is a very optimistic expectation (Block, 2004). Further, although this period of exposure may be required even for the most effective chlorine-releasing agents (Wheeldon *et al.*, 2008b), prolonged contact with NaDCC agents are not realistic due to the odour, corrosive effects on surfaces and irritation to users (Ungurs *et al.*, 2011). To further understand the cleaning of surfaces, it would be useful to perform similar tests in the presence of dried organic matter on the surfaces and perhaps, extend the studies to porous surfaces like wood and fabric. Interestingly, at 10 minutes on all the surfaces, the lower $\log_{10}$ reduction was observed for the epidemic ribotypes 027, 001 and 106.

The exposure of *C. difficile* spores to sub-MIC concentrations of disinfectants is a realistic possibility, but whether the sporulation capacity of the strains *in vivo* following this exposure changes remains unknown. In this study, the growth of all the strains was only markedly reduced by sub-MIC levels of TriGene Advance; this was perhaps due to a bacteriostatic effect which then resulted in no spores being detected in these cultures. In the presence of the other non-chlorine-based agents, ribotype 001 produced considerably more spores than ribotypes 027 and 106; however, these numbers were much greater than that of strain 630. The addition of sodium taurocholate to the test medium did not affect the growth of the strains but did positively affect the number of spores detected. The increased sporulation of ribotype 001 in the presence of sub-inhibitory concentrations of non-chlorine disinfectants has been documented and the data here are in agreement with this observation for not only ribotype 001 but also the other epidemic ribotypes 027 and 106. This observation could potentially explain the wider dissemination of these strains in the nosocomial environment but further studies have to be performed to determine the clinical significance of such exposure, not just in terms of environmental contamination, but also with regards to the possibly altered in-vivo virulence of these strains.

Evidence for the widespread contamination of the laboratory environment with *C. difficile* has been shown in this study and it is equally important to control this spread in laboratories due to the potential of laboratory-acquired CDI. Although the level of contamination was relatively high in the small area tested, it could possibly be higher; perhaps, if the detection medium contained sodium taurocholate or lysozyme which enhance germination (Wilson *et al.*, 1982) more contamination may have been detected. Also, as this sampling was performed only once, it was not possible to determine if this contamination is constant or transient. Regular sampling of surfaces and trials with different cleaning regimens would be useful. The sampling of commonly-used equipment would be important since the contamination of these, as observed in this study, can expose other workers to *C. difficile* who might not be aware of the risks of CDI, especially the antibiotic aspect.

The need for effective biocides against *C. difficile*, especially its spores, is clear. However, until an effective method for decontamination is developed, regular assessment of contamination in laboratories should be performed to determine the areas most prone to contamination. It has been shown in hospitals that frequent contact sites can be heavily contaminated (Fawley *et al.*, 2005). In the same study, approximately 14% of high-reach environmental sites which were not cleaned often were found to be *C. difficile*-positive. These sites, like shelves, were found to be contaminated in our laboratory as well and may act as reservoirs from where spore transfer can potentially take place after disturbance of air due to activity. *C. difficile* has been commonly but sporadically isolated from air in hospitals, mainly during peak activity times such as ward rounds, visiting hours and trolley services and during activities such as opening doors and bed-making (Best *et al.*, 2010). Also, the strains found causing disease were related to those isolated from the environment and air. The acquisition of *C. difficile* from the environment and air by the host has been documented using animal faecal-oral transmission models (Larson *et al.*, 1980; Lawley *et al.*, 2010). It is possible that there is similar dissemination of *C. difficile* across a laboratory and sampling the surfaces and air within a laboratory actively doing *C. difficile* research would give a good measure of the degree of spread. Clean

surfaces and clean air are very important from an environmental perspective; lowest aerial contamination is associated directly with the best ventilation, the least human activity and the most intensive housekeeping (Greene *et al.*, 1962b).

When testing for disinfectant activity, most European standards require a biocide to cause a 5 $\log_{10}$ reduction (Fraise, 2011), a norm that may be too rigorous and could lead to over-use of disinfectants (van Klingeren, 1995). In this study, approximately 10^3 spores were used to contaminate the test surfaces, but the environment might not be as heavily contaminated and thus, may be more efficiently cleaned. Also, unlike vegetative cells, preparing such concentrated spore suspensions of *C. difficile* are difficult at best (Fraise, 2011). However, in areas of intensive *C. difficile* work, contamination of surfaces by high concentrations of spores is a possibility and thus, tests with large concentrations of spores in the presence of organic matter and even faecal material should be performed. Also, along with the biocides themselves, the method of cleaning must also be evaluated as it has been observed that wiping of surfaces with the wrong agents can cause cross-contamination (Dharan *et al.*, 1999; Ungurs *et al.*, 2011).

The contaminated environment is important in the transmission of *C. difficile* and just as in hospitals, laboratories can be a source of transmission of CDI, due to both the virulent types of strains and high concentrations of *C. difficile* routinely present. Reports of laboratory-acquired CDI led to this evaluation of the type of disinfectants and cleaning agents used in our laboratory. From these studies, it would appear that chlorine-releasing agents should be the decontaminants of choice in laboratories, just as in hospitals (Fraise, 2011) although care has to be taken while using them owing to their possible toxic and corrosive side-effects and their reduced efficacy on soiled surfaces (Ungurs *et al.*, 2011).

The battle against *C. difficile* must be extended to the laboratory as it can be a source of disease transmission. The use of safety cabinets and disposable equipment, education and training of staff and the rigorous decontamination of surfaces are all required to reduce the threat of laboratory-acquired CDI. As suggested before,

cleaning the environment with detergents reduces the load of contamination but effective, user-friendly sporicidal agents are needed to tackle the spread of *C. difficile* (Wilcox, 1996). An agent that is sporicidal, effective at high contamination levels on vegetative cells on moist surfaces and spores dried onto surfaces in a practicable time, despite preparation in hard water and presence of organic material, and which is also non-corrosive and non-toxic would be the ideal biocide to rid any area of *C. difficile* (Kelsey *et al.*, 1974; McDonnell & Pretzer, 2001). Until such time as an effective method is developed to destroy *C. difficile*, basic hygiene must be maintained as described in the following poem (Donskey, 2010):

How many times must a doctor be told

Wash your hands and wear gloves, please?

Yes, and how many times will another stand by

Pretending he just doesn't see?

And how many times must we remind

Those things that we touch must be cleaned?

The answer, my friend, is blowin' in the wind

The answer is blowin' in the wind.

5. Interactions of *C. difficile* with human cells

5.1. Introduction

The toxins and spores of *C. difficile* are its most important virulence factors. However, *C. difficile* expresses several surface-associated proteins that are also involved in the infection process. These proteins are highly immunogenic but, perhaps more importantly, they may participate in the very first step of infection: adherence to host cells. The interactions between virulence factors and host cells can potentially affect the outcome of disease. The immune response elicited by macrophages in response to surface-associated proteins and toxins of different *C. difficile* strains and the role of flagella and S-layer proteins in adherence to epithelial cells were investigated.

5.1.1. Surface-associated proteins

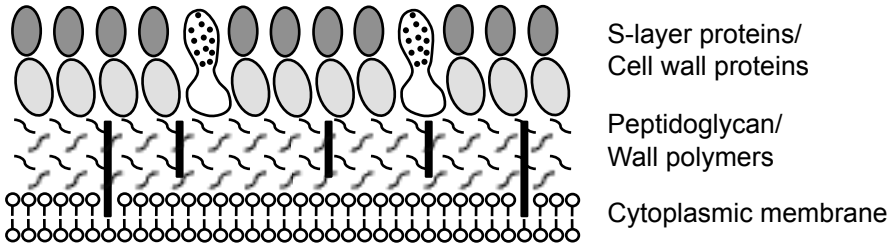
5.1.1.1. S-layer proteins

Surface-layers are porous crystalline membranes composed of identical proteins or glycoproteins which form the outermost envelope of some Gram-positive and Gram-negative bacteria (Sidhu & Olsen, 1997; Sleytr & Messner, 1993). They have a variety of functions such as maintenance of the shape and rigidity of the cell envelope, molecular sieving, protection against complement-killing, interaction with macrophages and generation of an immune response and adhesion and colonisation. They can even be essential for virulence in some bacteria (Sára & Sleytr, 2000).

In most bacterial species, the S-layer is composed of a single glycosylated protein (Sára & Sleytr, 2000) but in *C. difficile*, it is composed of two S-layer proteins (SLPs) - a surface-exposed protein with a lower molecular weight between 32 and 38 kDa (LMW SLP) and a cell wall-associated protein with a higher molecular weight between 42 and 48 kDa (HMW SLP) (Fig. 5.1) (Fagan *et al.*, 2009; Takeoka *et al.*, 1991). Using techniques such as freeze-etching and negative staining, it was determined that the S-layers in *C. difficile* had a distinct arrangement; the two protein

subunits were structurally superimposed on one another with the outer LMW SLP layer showing square symmetry and the inner HMW SLP layer showing hexagonal symmetry (Cerquetti *et al.*, 2000; Takeoka *et al.*, 1991). Also, it was initially suggested that *C. difficile* SLPs were glycosylated owing to the disparity between the observed and predicted molecular weights of the two proteins (Cerquetti *et al.*, 2000). Indeed, in one study the glycosylation of HMW SLPs of all the strains investigated and of some LMW SLPs of the same strains was observed (Calabi *et al.*, 2001). However, more recent data have now confirmed that *C. difficile* SLPs are not glycosylated and therefore, assembly of this unique S-layer is not dependent on glycosylation but on non-covalent interactions between the SLPs (Qazi *et al.*, 2009).

Fig. 5.1. Model of the cell wall of *C. difficile*

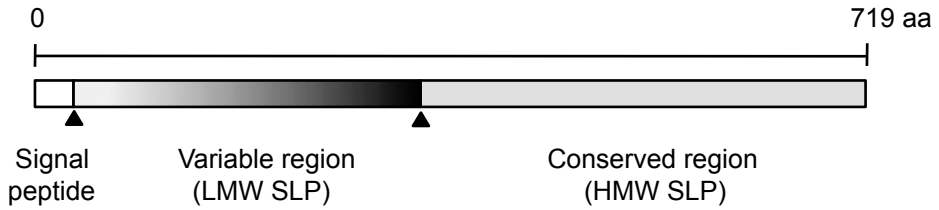


In the cell wall of *C. difficile*, the two SLP subunits are arranged above the peptidoglycan layer. The HMW SLP (light grey) is embedded in the wall while the LMW SLP (dark grey) is surface-exposed. Other cell-wall proteins are also found above the peptidoglycan layer. Adapted from Fagan *et al.*, 2009.

The unique 2160 bp *slpA* gene codes for the 73.4 kDa SlpA precursor protein (Fig. 5.2) (Karjalainen *et al.*, 2001; Karjalainen *et al.*, 2002). SlpA has a two-domain structure characteristic of surface-expressed proteins. It has a signal sequence at the N-terminal and a C-terminal with three imperfect intermolecular repeat sequences (Karjalainen *et al.*, 2001). The S-layer homologous (SLH) domain which anchors the SLPs to secondary cell wall polymers in most bacteria is not found in *C. difficile* (Calabi *et al.*, 2001; Mukherjee *et al.*, 2002). The maturation of SlpA is required for the generation of the mature LMW SLP and HMW SLP subunits (Karjalainen *et al.*, 2001; Mukherjee *et al.*, 2002). Post-translational cleavage of the precursor was found

to occur at two sites - at the N-terminal to remove the signal peptide and internally at approximately position 355 to separate the two protein subunits (Calabi *et al.*, 2001). It is for this reason that only one band was observed when SLPs extracted from *C. difficile* strains were run on a native protein gel, whereas two distinct bands were observed under reducing conditions (Calabi *et al.*, 2001). Cleavage of the LMW SLP and the HMW SLP after removal of the signal peptide could be mediated by the cell wall protein (CWP) and cysteine protease Cwp84 (Kirby *et al.*, 2009). However, Cwp84 was not found to be essential for this process and maturation of the proteins occurred even through the action of extracellular trypsin and possibly occurs directly through host gut proteases *in vivo*.

Fig. 5.2. Structural organisation of the SlpA precursor protein of *C. difficile*



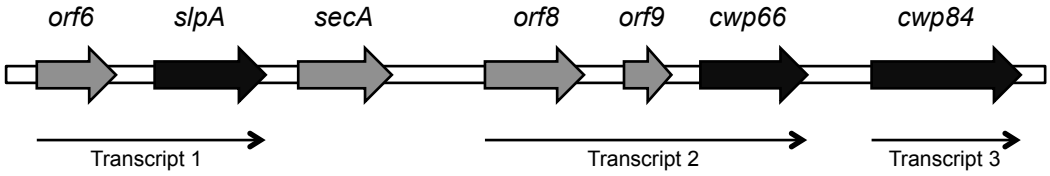
The SlpA precursor protein undergoes cleavage twice - once to remove the signal peptide and again to generate the HMW SLP and the LMW SLP subunits. The cysteine protease Cwp84 can mediate this cleavage, although this action is not specific; extracellular trypsin and chymotrypsin are also able to cleave the SlpA precursor into its two subunits. Adapted from Karjalainen *et al.*, 2002 and Fagan *et al.*, 2009.

Analysis of SlpA showed that the N-terminal of the protein corresponded to the LMW SLP while the C-terminal corresponded to the HMW SLP (Karjalainen *et al.*, 2001). Several variations were observed in the N-terminal domain, while the C-terminal was more conserved. The role of the N-terminal in adherence to epithelial cells was detected and it was thus suggested that the variability observed in the N-terminal was due to antigenic selection owing to its exposure on the bacterial surface. The C-terminal showed homology with autolysins LytC (CwlB) and LytB of *Bacillus subtilis* as well as the N-terminal of Cwp66, which also exhibits N-acetylmuramoyl-

L-alanine amidase activity (Calabi *et al.*, 2001; Karjalainen *et al.*, 2001; Mukherjee *et al.*, 2002). Investigations with several strains showed that there was no homology between the two SLPs of the same strain suggesting that each protein had a unique function (Calabi *et al.*, 2001; Cerquetti *et al.*, 2000). Further, very little homology was observed between the N-termini of the proteins between different strains. This high inter-strain variability in sequences and molecular weights of the SLPs between strains has formed the basis of genetic and phenotypic typing schemes (Kato *et al.*, 2010; McCoubrey *et al.*, 2003; Mukherjee *et al.*, 2002). The signal sequences of SlpA proteins, however, are highly conserved between strains (Calabi *et al.*, 2001). They have been identified as Sec-dependent signal peptides and indicate the role of *secA* in the transport of the SLPs across the cell membrane (Mukherjee *et al.*, 2002). The limited variability in the HMW SLPs of different *C. difficile* strains was found to be a result of sequence insertion rather than point mutations (Calabi *et al.*, 2001). Only two conserved motifs were identified in the LMW SLP spanning the C-terminal residues 245 to 274 and 304 to 321, respectively (Calabi & Fairweather, 2002; Fagan & Fairweather, 2010). These are essential for interaction of the LMW SLP with the HMW SLP to form an 'end-to-end' complex in the S-layer.

slpA is encoded in a region of the *C. difficile* genome which contains at least 28 paralogues coding for proteins with homology to the HMW SLP (Fig. 5.3) (Calabi *et al.*, 2001; Calabi & Fairweather, 2002). These genes and their intergenic regions appear to be fairly conserved across strains (Mukherjee *et al.*, 2002). Together these results suggest that this gene cluster is involved in cell wall synthesis. *secA* is located immediately downstream of *slpA*. Simultaneous expression of these genes was observed during growth (Calabi *et al.*, 2001). *slpA* was transcribed throughout growth from the early exponential into the stationary phase (Savariau-Lacomme *et al.*, 2003). The 3.2 kb transcript observed proved co-transcription with *or6f*. This correlated well with the abundance of SLPs detected extracellularly during the growth of *C. difficile* (Mukherjee *et al.*, 2002).

Fig. 5.3. Genetic organisation of part of the DNA cluster in *C. difficile* strain 630 that includes *slpA*, *cwp66* and *cwp84*



The genes encoding surface-associated proteins in *C. difficile* are arranged close to one another on the genome and are transcribed in the same direction. *slpA* is transcribed bicistronically with *orf6* throughout growth. Polycistronic and monocistronic transcription of *cwp66* and *cwp84*, respectively, is only observed in the exponential phase of growth. The *secA* gene is immediately downstream of *slpA*. Adapted from Savariau-Lacomme *et al.*, 2003 and Calabi *et al.*, 2001.

The SLPs of *C. difficile* are undoubtedly immunogenic. The HMW SLP is conserved and therefore shows strong antigenic cross-reactivity with antisera to a variety of strains (Calabi *et al.*, 2001; Cerquetti *et al.*, 2000) but the LMW SLP is the dominant antigen (Cerquetti *et al.*, 1992). It has been observed that patients with recurrent disease have lower IgM to SLPs than those with a single episode (Drudy *et al.*, 2004). In fact, SLPs have been identified as useful candidates for active and passive immunisation in the hamster model (Ní Eidhin *et al.*, 2008; O'Brien *et al.*, 2005).

C. difficile SLPs also play a role in adherence. SLPs from three *C. difficile* strains were all found to bind similarly to HEp-2 cells (Calabi *et al.*, 2002). In the same study, using recombinant SLPs it was shown that there was stronger adhesion with the HMW SLP than there was with the LMW SLP. The use of anti-HMW SLP antiserum reduced adherence of bacterial cells to epithelial cells by approximately 20 to 30%, while pre-incubation of recombinant HMW SLP with antiserum prevented any inhibition, thus confirming the role of the HMW SLP in binding. Further, adherence of native and recombinant SLPs to normal mucosal biopsy specimens from the human gastrointestinal tract was investigated. Both protein types were able to adhere to epithelium and lamina propria from the stomach, duodenum and colon. Maximum binding was observed on apical surfaces of epithelial cells suggesting the

presence of receptors for SLPs on the brush border. Adherence was also strongest to the apexes of villi indicating that the state of differentiation of host cells might be important for this interaction. The proteins also adhered to the extracellular matrix (ECM) in particular, to the proteins collagen I, thrombospondin and vitronectin. The HMW SLP showed equivalent binding as compared to the native protein, while the LMW SLP attached to only a few surface epithelial cells.

It was hypothesised that specific adherence of bacteria to the brush border membrane through SLPs could allow for targeted delivery of toxins and that the subsequent destruction of the epithelium and ECM could enhance further damage and possibly aid in evasion from the immune system (Calabi *et al.*, 2002). Recombination of *slpA* genes between strains has also been suggested as a mechanism to evade the immune system of the host (Ní Eidhin *et al.*, 2006).

5.1.1.2. Flagella

Bacterial flagella are sensory and motor surface organelles (Macnab, 2003). They are required for motility, adhesion and invasion of mucosal surfaces and interact directly with the immune system of the host (Ramos *et al.*, 2004). Flagella are present on the surface of most *C. difficile* strains but non-flagellated strains do exist (Delmée *et al.*, 1990). However, flagellum proteins have been purified and the encoding genes have been amplified from seemingly non-motile strains suggesting that the expression of flagella could be phase-variable (Pituch *et al.*, 2002; Twine *et al.*, 2009). It has also been proposed that translation of flagellum proteins may be regulated by environmental signals and that strains that appear non-motile *in vitro* may very well be motile *in vivo* (Tasteyre *et al.*, 2000b).

When *C. difficile* flagella were first identified, the 39 kDa protein was found to be similar in all flagellated strains and thus, was found to be responsible for cross-agglutination observed in serogrouping reactions (Delmée *et al.*, 1990). Also, the presence or absence of flagella had no correlation with toxin production and hence, was not considered to be a virulence determinant. However, this did not imply that flagella had no role in adherence or colonisation.

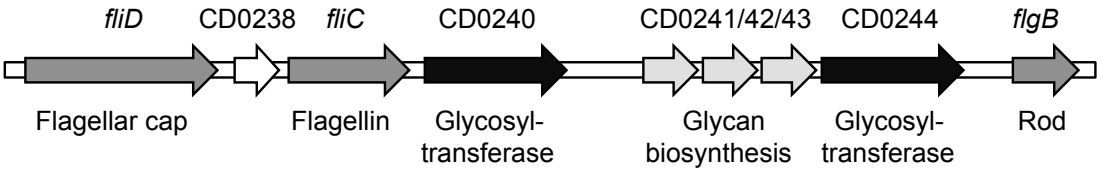
The *fliC* gene codes for the flagellin filament, while the *fliD* gene codes for the flagellar cap. *fliC* is a gene of 870 bp and the FliC protein is composed of 290 amino acid residues (Tasteyre *et al.*, 2000a). Only one copy of *fliC* is found in the *C. difficile* genome. The flagella have the characteristic three repetitive domains found in surface-exposed proteins. They also have structural features commonly found in other flagellin proteins. FliC has several conserved alanine residues which are responsible for the mainly α-helical conformation of the filament. It also shows the presence of a LIAN sequence used for export of flagellar subunits. The N- and C-terminals of FliC which are responsible for secretion and polymerisation, respectively, are conserved. The central region is divergent between strains as is it surface-exposed and thus, undergoes selection by antigenic drift. It is responsible for cross-reactivity between strains, which hinders typing methods like serogrouping (Delmée *et al.*, 1990). However, the genetic differences are sufficient to be applied to a typing method involving RFLP analysis of *fliC* (Tasteyre *et al.*, 2000a; Tasteyre *et al.*, 2000b). Interestingly, in one study high conservation of FliC was observed between clinical strains isolated over a short period of time (Twine *et al.*, 2009).

fliD is a 1524 bp gene that codes for the FliD cap protein (Tasteyre *et al.*, 2001b). Just like *fliC*, there is only copy of *fliD* in the *C. difficile* genome. FliD is 56 kDa protein composed of 507 amino acids. FliD is also surface-exposed, but unlike FliC, it is highly conserved and consists of no variable domains, suggesting it has a very specific function in adherence to cell or mucus receptors. On treatment with a range of restriction endonucleases *fliD* shows two main RFLP patterns further illustrating the conservation of its genetic sequence. Translational variation observed for FliC in phenotypically non-flagellated strains has been observed for FliD as well.

In initial studies, the predicted molecular weight of *C. difficile* flagella was different from the observed weight but glucosylation was not detected suggesting alternate post-translational modification of the protein (Tasteyre *et al.*, 2000a). In a more recent study, a discrepancy of approximately 398 Da was detected, which indicated the addition of a glycan molecule (Twine *et al.*, 2009). This was confirmed by mass

spectrometry and O-linked glycan units were detected on the flagella. Further, a glycosyltransferase CD0240 was detected immediately downstream of *fliC* along with other glycosyltransferases and genes involved in glycan biosynthesis (Fig. 5.4). The presence of CD0240 was found to be essential for flagellin production; interruption of the gene resulted in suboptimal production of flagellin. This confirmed that *C. difficile* flagella are glycoproteins. This made *C. difficile* the first example of a Gram-positive bacterium in which glycosylation was required for the assembly of flagella and subsequent motility.

Fig. 5.4. Genetic organisation of part of the DNA cluster in *C. difficile* strain 630 that codes for the flagella proteins



The genes encoding the different flagellar proteins are arranged in close proximity on the *C. difficile* genome. Of these, *fliD* codes for the flagellar cap, *fliC* codes for the flagellin filament and *flgB* codes for the flagellum rod. *C. difficile* flagella are glycosylated by glycosyl-transferases such as CD0240 and CD0244 which are responsible for the addition of glycan units to flagella by O-linkages. Adapted from Twine *et al.*, 2009.

Just like other surface proteins, flagella interact with the immune system of the host. Antibodies to FliC and FliD have been detected in patient sera, although FliD was found to be more immunogenic (Péchiné *et al.*, 2005a). Unsurprisingly, *C. difficile* flagella are also involved in adherence. The adherence of FliC, FliD and crude flagella to mucous membranes was investigated (Tasteyre *et al.*, 2001a). All three protein preparations bound to the axenic caecal mucus from mice but not to porcine stomach mucus suggesting the absence of receptors for *C. difficile* flagella in pigs. However, the role of flagella in adherence to mucus was confirmed. When attachment of the proteins to Vero cells was studied, no adhesion was observed with FliC, intermediate adhesion was observed with crude flagella and maximum

adhesion was observed with FliD. No binding was seen under denatured conditions. Thus, the native conformation of FliD is important in binding. Overall, FliC and FliD were both involved in attachment to mucus. In mice, no difference in colonisation was detected between flagellated and non-flagellated strains but flagellated strains showed greater adherence to mouse caeca *in vivo*. The presence of toxin B enhanced both adherence and colonisation; the damage caused to host cells by toxin B possibly led to the exposure of additional receptors, which could be bound by flagella.

5.1.1.3. GroEL

Heat-shock proteins (HSPs) in bacteria are highly conserved stress-inducible constitutive proteins which are involved in maintaining homeostasis within the bacterial cells (Zügel & Kaufmann, 1999). These molecular chaperones are important antigens in humoral and cellular immune responses elicited in the host.

GroEL is a HSP expressed by *C. difficile* belonging to the Hsp60 family (Hennequin *et al.*, 2001b). It is encoded on the 1940 bp *groESL* operon. A single copy of the operon exists on the genome and it appears to be positively and negatively regulated. The operon includes the 1632 bp *groEL* gene and the 285 bp *groES* gene which code for 58 kDa and 10 kDa proteins, respectively. Neither protein has a signal peptide and both have a predominantly α-helical structure with one potential transmembrane domain. GroEL is highly conserved between different *C. difficile* strains.

The expression of GroEL is strongly induced following heat-shock above 40°C (Hennequin *et al.*, 2001a; Hennequin *et al.*, 2001b). Transcription of *groEL* was found to increase rapidly after heat-shock at 43°C for five minutes and reached a plateau within an hour. After this treatment, a 3-fold increase in protein production was detected (Hennequin *et al.*, 2001a). A large amount of GroEL was found in the cell membrane fraction and extracellularly. The increase in *groEL* transcription was less prominent at 48°C; however, GroEL was detected in the cytoplasmic and membrane fractions and also in the cell wall fraction (Hennequin *et al.*, 2001b). It was uniformly distributed over the cell surface and also found in the extracellular space. Further, conditions of stress such as acidic environment, iron-deprivation or

high osmolarity induced significant transcription and translation of this HSP (Hennequin *et al.*, 2001a). The presence of ampicillin caused a significant increase in *groEL* transcription, but slower translation of the protein. This suggested that different stresses had different effects on GroEL expression.

The mechanism of transport and localisation of GroEL to the surface of *C. difficile* cells and into the extracellular space is unknown. Since GroEL lacks a signal peptide, its transport into the extracellular space was hypothesised to occur by surface adsorption to the extracellular surface of the cell wall, followed by release of the protein (Hennequin *et al.*, 2001b). Surface-exposure of GroEL was thought to be necessary for its interaction with host cells via its hydrophobic domains. GroEL was found to adhere to Vero cells and its role as an adhesin was confirmed when this attachment was inhibited by antibodies to GroEL and purified GroEL protein. Interestingly, GroEL expression was also induced on contact with Vero cells under normal temperature conditions indicating that its role in adhesion is not limited to conditions of stress (Hennequin *et al.*, 2001b).

5.1.1.4. Cwp66

Cwp66 (cell wall protein 66 kDa) was detected in *C. difficile* following heat-shock at 60°C and was the first adhesin to be identified in a *Clostridium* species (Waligora *et al.*, 2001). It is encoded by the *cwp66* gene which lies in the same operon-like region of the genome as *slpA* (Fig. 5.3). This 1830 bp gene is unique. *cwp66* expression occurs only in the early exponential phase of growth; it is polycistronically transcribed as a 5.5 kb transcript along with *orf8* and *or9f*, which lie immediately upstream of it (Savariau-Lacomme *et al.*, 2003). *orf9* encodes a 24.9 kDa hydrophilic protein with no homology to any known protein (Waligora *et al.*, 2001). Polycistronic transcription of *cwp66, orf9 and orf8* suggests that the protein products of the three genes together may form an adhesin (Savariau-Lacomme *et al.*, 2003).

Cwp66 also shows homology to CwlB of *B. subtilis*, like the SLPs (Waligora *et al.*, 2001). Cwp66 has a surface-exposed C-terminal, a mainly α-helical N-terminal and three imperfect intramolecular repeat motifs characteristic of surface-exposed

proteins. Although variations were observed in the N- and C-terminals of the protein, the latter is more variable possibly owing to its surface-exposure and subsequent immune selection. Several nucleotide changes have been identified in the C-terminal domain of Cwp66 which result in at least nine peptide sequences (Savariau-Lacomme *et al.*, 2003). Based on variations in Cwp66, three groups of *C. difficile* strains were identified: those with no variability in the entire protein, those with differences only in the C-terminal and those with variations in both terminals (Waligora *et al.*, 2001). A cleavage site between Ala27 and Ser28 suggested that the mature Cwp66 protein is exported to the cell membrane or secreted; however, Cwp66 does not have any transmembrane domains or cell wall-anchoring motifs (Waligora *et al.*, 2001).

Antibodies generated against the N- and C-terminals of Cwp66 were observed to react with proteins in the cytoplasm, cell membranes and cell walls of *C. difficile* (Waligora *et al.*, 2001). Anti-Cwp66-N antibody detected very few proteins in heat-shocked and non-heat-shocked bacteria suggesting that this terminal was embedded in the cell wall. Anti-Cwp66-C antibody detected a few proteins in non-heat-shocked bacteria. However, in heat-shocked bacteria, large amounts of protein were detected evenly distributed over the cell-surface, using the anti-Cwp66-C antibody, thus confirming that the C-terminal is surface-exposed and possibly involved in adherence. The role of Cwp66 in attachment was subsequently investigated by Waligora and colleagues; partial inhibition of adhesion to Vero cells was observed with antibodies to Cwp66 and purified protein but only for heat-shocked bacteria. This partial inhibition indicated the presence of several other adhesins in *C. difficile*.

5.1.2. Gut-associated lymphoid tissue

The gastrointestinal (GI) tract encounters a vast number of varied microbial antigens and the immune system of the GI tract, the gut-associated lymphoid tissue (GALT), has the daunting task of differentiating between commensal and pathogenic organisms - providing immune tolerance to the former and protection from the latter (Mason *et al.*, 2008). The GALT is the largest compartment of the immune system

and includes the Peyer's patches, mesenteric lymph nodes and isolated lymphoid follicles (Platt & Mowat, 2008). A large variety of immune cells including macrophages, dendritic cells, activated T cells, mast cells and plasma cells together maintain immunological homeostasis in the gut through the rapid elimination of pathogens by phagocytosis and enhanced production of secretory IgA (sIgA) which can mediate antigen neutralisation and bacterial phagocytosis, while inducing anti-inflammatory Th2 responses and suppressing inflammatory Th1 responses (Fig. 5.5) (Mason *et al.*, 2008).

Antigen recognition by the immune system occurs through pathogen recognition receptors (PRRs) such as toll-like receptors (TLRs), which recognise pathogen-associated molecular patterns (PAMPs), for example TLR-4 recognises LPS (Platt & Mowat, 2008; Sherman & Kalman, 2004). Lumenal antigens are sampled by M cells and dendritic cells which send dendrites into the lumen (Sherman & Kalman, 2004). On identification and differentiation, the immune response generated either leads to homeostasis or inflammation. Intestinal macrophages are crucial in these responses (Weber *et al.*, 2009).

Monocytes, the precursors of macrophages and dendritic cells, are produced in the bone marrow (Smith *et al.*, 2005; Weber *et al.*, 2009). After circulating in the blood for several days, they reach the target tissues where they differentiate into macrophages. The intestinal mucosa is the largest reservoir of macrophages. Most of these macrophages are ideally located in the sub-epithelium and are often referred to as 'resident macrophages'. They differ in phenotype and function from macrophages found in other tissues (Platt & Mowat, 2008; Smith *et al.*, 2005; Weber *et al.*, 2009). They have increased cytoplasmic volume, distinct pseudopodia and prominent vacuoles and secondary lysosomes. They do not proliferate in the intestine and appear to be in a state of partial activity. They exhibit low or no expression of TLRs, Fc receptors for IgA and IgG, complement receptors and receptors for pro-inflammatory cytokines. They do not produce pro-inflammatory cytokines such as TNF-α, IL-1 or IL-6 and only produce low levels of IL-8. Instead, they produce

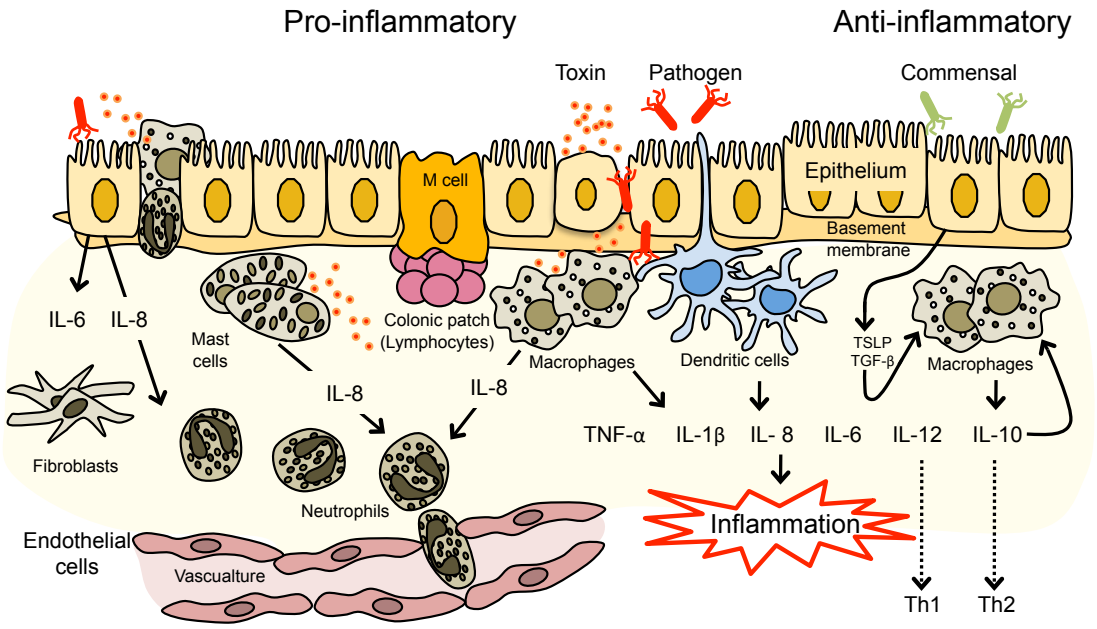
IL-10 and drive anti-inflammatory Th2 responses in the gut. These alterations, however, do not affect their phagocytic potential (Platt & Mowat, 2008).

The characteristic mucosal phenotype of intestinal macrophages appears to be a response to the microenvironment of the gut (Platt & Mowat, 2008; Weber *et al.*, 2009). The production of soluble factors, especially IL-10 and TGF-β, by other intestinal cells has been found to induce this phenotype. Phagocytosis of apoptotic cells induces the production of IL-10 in macrophages which could also be a factor. However, direct contact with epithelial cells was found to be crucial for this effect (Weber *et al.*, 2009). It has been observed that mucosal dendritic cells (DCs) also uniquely drive a Th2 response and induce B cells to produce sIgA to maintain non-inflammatory conditions in the gut (Rimoldi *et al.*, 2005b). Mucosal DCs appeared to be conditioned through stimulation with epithelial cell-derived medium containing thymic stromal lymphopoeitin (TSLP). They failed to produce IL-12 in response to bacteria and thus, were unable to drive a Th1 response, but they did produce IL-6 which induced IgA production by plasma cells. Since intestinal macrophages are physically separated from epithelial cells by the basement membrane, the mechanism of the interaction between intestinal macrophages and epithelial cells is unknown; interaction could occur if intestinal macrophages sent out protrusions through the epithelium similar to the dendrites of dendritic cells (Weber *et al.*, 2009).

The mucosal phenotype of intestinal macrophages does not necessarily imply that they have lost the ability to induce pro-inflammatory cytokines (Weber *et al.*, 2009). During an infection they can induce TNF-α production upon TLR stimulation. Intestinal inflammation is characterised by the destruction of epithelial cells, the production of Th1-type cytokines, crypt abscesses and eventual loss of barrier function (Sherman & Kalman, 2004). In *C. difficile* infection, activated macrophages release TNF-α and other inflammatory mediators (Castagliuolo *et al.*, 1997). There is also degranulation of mast cells, neutrophil infiltration, increased epithelial permeability and fluid secretion (Wershil & Castagliuolo, 1998). In such an infection, macrophages and DCs previously conditioned by epithelial cells to drive a

Th2 response can produce pro-inflammatory cytokines (Eckmann & Kagnoff, 2005; Platt & Mowat, 2008). However, it has also been suggested that only newly recruited DCs and macrophages that have not been pre-conditioned by epithelial cells are able to induce the required protective Th1 response (Rimoldi *et al.*, 2005b).

Fig. 5.5. Immune system of the intestine: its role in infection and health



In the intestine, a variety of immune cells act together provide protection from potential pathogens. During infection or inflammation, invasive pathogens or their products can activate the intestinal epithelial cells and immune cells such as macrophages to secrete pro-inflammatory cytokines like TNF-α, IL-1, IL-8 and IL-12, resulting in the recruitment of neutrophils, monocytes and other inflammatory cells, which can eventually result in hyper-responsiveness of the immune system and chronic inflammation. Such inflammation, involving the influx of large numbers of neutrophils, loss of epithelial barrier function, fluid accumulation and ulceration is observed in infection with *C. difficile*. Under non-infection conditions, intestinal macrophages are deficient in TLRs and other pro-inflammatory receptors and are hypo-responsive but maintain their phagocytic activity. Mediators such as TSLP, IL-10 and TGF-β released by other cells and IL-10 released by macrophages themselves condition them to drive anti-inflammatory responses and maintain homeostasis. Adapted from Platt *et al.*, 2008, Sherman *et al.*, 2004, Eckmann *et al.*, 2005, Rimoldi *et al.*, 2005 and Poxton *et al.*, 2001.

In an inflammatory setting, macrophages can contribute to tissue pathology by the recruitment of neutrophils through the production of large amounts of IL-8 and by TNF-α meditated up-regulation of adhesion molecules on blood vessels that aid this process (Platt & Mowat, 2008). IL-12 produced by the macrophages induces IFN-γ production by T cells, which results in increased epithelial permeability. Further, TNF-α and IL-1 drive apoptosis of epithelial cells, barrier disruption, vascular damage and necrosis and can also lead to the degradation of collagen and other extracellular matrix proteins. It is not known whether the macrophages that drive this Th1 response are resident intestinal macrophages in which functional reversion has occurred or non-mucosal macrophages recruited to the site of inflammation. This increased responsiveness by the GALT results in chronic inflammation and ulceration (Platt & Mowat, 2008).

5.1.3. Immune response in CDI

Intestinal inflammation is a common pathological outcome of *Clostridium difficile* infection, which is associated with pseudomembrane formation. Pseudomembranes are formed of unevenly distributed 'volcano-like' inflammatory lesions. These lesions consist of sloughed epithelial cells, an array of immune cells, especially neutrophils, and fibrin and lie over mucosa which also shows ulcerations consisting of necrotic epithelial cells and marked leukocytic infiltration of the lamina propria (Castagliuolo & LaMont, 1999; Hafiz & Oakley, 1976; Knoop *et al.*, 1993). This acute inflammatory response is thought to contribute to tissue damage and injury and directly affect the clinical severity of disease (Savidge *et al.*, 2003). Greater levels of faecal IL-8 and IL-1β have been detected in patients with moderate to severe CDI as compared to patients with mild disease, highlighting this correlation (Steiner *et al.*, 1997).

The role of the toxins of *C. difficile* in inflammation has been widely investigated. Early studies showed that *C. difficile* toxins could inhibit monocyte-dependent T cell proliferation suggesting that monocytes were the primary targets of the toxins (Däubener *et al.*, 1988). Treatment of monocytes with toxin B resulted in the

production of pro-inflammatory cytokines like IL-1α, IL-1β, IL-6 and TNF-α (Flegel *et al.*, 1991). Toxin A was also able to elicit a similar response but at a 1000-fold higher dose. Moreover, a certain level of synergism between the toxins and LPS was observed in the production of these cytokines. In fact, small amounts of toxin A that were insufficient to elicit a response were able to induce cytokine production in the presence of LPS. No synergism was observed between the two toxins. At low concentrations of toxin B, there was a slow and sustained release of IL-1β, while at higher concentrations, there was an immediate release of IL-1β. Even a short pulse with large amounts of toxin B was able to induce a high IL-1β response in the monocytes, even though the toxin is highly cytotoxic. The induction of these pro-inflammatory responses by the toxins suggested that the responses themselves could contribute to the damage characteristic of CDI. In another study, monocyte-toxicity due to toxin B was not observed but the toxin did induce TNF-α release, alter the morphology of the monocytes and affect phagocytosis (Siffert *et al.*, 1993). The toxins were also found to induce the production of large amounts of TNF-α by macrophages (Linevsky *et al.*, 1997; Melo Filho *et al.*, 1997). Toxin B was found to be more effective at inducing TNF-α production and more cytotoxic to macrophages than toxin A. Both toxins have been shown to be more cytotoxic to macrophages and monocytes as compared to T and B cells (Mahida *et al.*, 1998) and macrophages were more sensitive than monocytes (Linevsky *et al.*, 1997).

The toxins are also cytotoxic to epithelial cells (Mahida *et al.*, 1996) which respond by producing IL-6 (Ng *et al.*, 2003); IL-6 production was enhanced by TNF-α and IL-1β (Ng *et al.*, 2003). Apical exposure of epithelial cells to the toxins has been seen to alter membrane permeability resulting in a loss of epithelial barrier function (Canny *et al.*, 2006; Sutton *et al.*, 2008). Exposure to the toxins has been observed to cause increased expression of IL-8 by epithelial cells and macrophages (Canny *et al.*, 2006; Johal *et al.*, 2004c; Savidge *et al.*, 2003). Disruption of the surface epithelium is likely to allow both *C. difficile* cells and toxins to reach the lamina propria and induce deeper damage (Johal *et al.*, 2004b). Neural responses have also been observed in response to the toxins. Toxin A has been seen to induce the production of

substance P by lamina propria macrophages in a time-dependent manner; substance P can stimulate the production of TNF-α and IL-1 by macrophages and also interact with other immune cells (Castagliuolo *et al.*, 1997).

One of the prominent features of inflammation in CDI is the high influx of neutrophils to the site of damage. Toxin A and toxin B both depend on macrophage-derived IL-8 to mediate neutrophil infiltration (Kelly *et al.*, 1994; Linevsky *et al.*, 1997; Souza *et al.*, 1997). Toxin A can directly affect neutrophil migration but only at very high concentrations, while toxin B does not do so, which makes macrophages key to the immune response in CDI. The production of TNF-α and IL-1β by toxin A-treated macrophages also induced neutrophil migration (Rocha *et al.*, 1997; Souza *et al.*, 1997). IL-8 up-regulates cell adhesion factors in neutrophils and in endothelial cells, thus enhancing the extravasation of neutrophils (Canny *et al.*, 2006; Linevsky *et al.*, 1997). Blocking the leukocyte adhesion molecule CD18 prevented neutrophil migration and toxin damage in toxin A-treated ileal loops confirming the importance of neutrophils in CDI (Kelly *et al.*, 1994). IL-8 induced neutrophil migration is also dependent on the presence of mast cells (Rocha *et al.*, 1997) which produce IL-8 and undergo slow controlled degranulation on toxin-exposure (Meyer *et al.*, 2007).

The C-terminal of toxin A was found to be capable of directly activating endothelial cells and mononucleocytes to release IL-8 and IL-6, which increased the expression of adhesion molecules in endothelial cells and facilitated neutrophil influx (Yeh *et al.*, 2008). The C-terminal of toxin B was also able to cause intestinal epithelial cell damage and stimulate IL-8 production (Zemljic *et al.*, 2010). Interestingly, the internalisation of the toxins into macrophages by endocytosis was crucial for the production of TNF-α and IL-1β (Ng *et al.*, 2010; Sun *et al.*, 2009). Disruption of the actin structure within the macrophages was not sufficient for the production of TNF-α and it was reliant on the glucosyltransferase activity of the toxins (Sun *et al.*, 2009). For IL-1β however, the activity of the toxins was not essential but the presence of the entire protein was (Ng *et al.*, 2010). Along with severe damage to the intestinal epithelium and induction of inflammatory cytokine responses, apoptosis of

macrophages, T-cells and eosinophils was also observed (Mahida *et al.*, 1996; Mahida *et al.*, 1998). Apoptosis was induced by caspase-dependent pathways (Brito *et al.*, 2002; Huelsenbeck *et al.*, 2007; Qa'Dan *et al.*, 2002). In this way, *C. difficile* toxins induce cytokine responses in a variety of cells that can further amplify the response inflammatory immune response.

Proteins of *C. difficile* other than its potent toxins can also elicit an immune response. A large number of proteins are released along with the toxins during growth of *C. difficile* (Mukherjee *et al.*, 2002). In fact, serum IgG to such surface proteins has been detected in patients and healthy adults in many studies (Mulligan *et al.*, 1993; Pantosti *et al.*, 1989; Sánchez-Hurtado *et al.*, 2008) and in many, a correlation between lower levels of antibodies to somatic antigens and the occurrence or recurrences of disease was identified (Kyne *et al.*, 2000; Mulligan *et al.*, 1993; Péchiné *et al.*, 2005a).

Several surface-associated proteins have been identified in *C. difficile* that can interact with the host immune system. A study aimed at identifying the immune response to these antigens in CDI patients found that patient serum reacted with SLPs, Cwp66, FliC, FliD and Fbp68 (Péchiné *et al.*, 2005a). Interestingly, in this study, the toxins appeared to be less immunogenic than the somatic antigens; differences among patients and controls did not lie in the immune response elicited by the toxins but only to that generated against the surface-adhesins. It was concluded that adhesins were able to induce a host immune response during the course of infection and the intensity of this response affected the outcome of infection. In another similar study, patient sera reacted with a variety of surface proteins (Wright *et al.*, 2008). SLPs were the most commonly recognised antigens and several paralogues of this protein were also recognised by patients. Antibody responses to the surface antigens were investigated on the day of diagnosis and at day 12 and in general, the levels of antibodies increased considerably at day 12 showing that there was exposure of the immune system to these surface antigens and

that patients were mounting a response to them. In patients where antibodies were detected from day 1 previous infection or carriage was suspected.

Studies with SLPs of *C. difficile in vitro* have identified them as strong immunogens that can cause monocytes to release large amounts of pro- and anti-inflammatory cytokines and thus, modulate induction of either a Th1 or a Th2 inflammatory response (Ausiello *et al.*, 2006; Bianco *et al.*, 2011).

5.1.4. Adherence in CDI

Common with most pathogenic bacteria adherence by *C. difficile* is a prerequisite to the infection and pathology (Drudy *et al.*, 2001). The importance of adherence in CDI was first demonstrated by Borriello and colleagues (Borriello *et al.*, 1988). They compared the ability of a highly toxigenic strain, a poorly toxigenic strain and a non-toxigenic strain to attach to the gut mucosa of hamsters. The highly toxigenic strain was most successful at mucosal colonisation, while the poorly toxigenic strain adhered to the mucosa as well as the non-toxigenic strain. Interestingly, the presence of toxin A and toxin B increased binding of the non-toxigenic strain. The toxigenic strains were found to be flagellated and motile, in contrast to the non-flagellated, non-motile non-toxigenic strain. It was suggested that the toxins exposed surface receptors or reduced the host's ability to prevent bacterial adherence. This study also suggested the existence of adhesins other than flagella in *C. difficile*.

Further investigations into adherence of *C. difficile* showed that the bacteria could attach to the enterocyte-like Caco-2 cells and the mucus-secreting HT29-MTX cells, but not to HeLa cells (Eveillard *et al.*, 1993). Bacterial adherence to Caco-2 cells was found to be limited to the apical mucosal surface and increased with time, reaching a maximum by two hours. Adhesion also varied with age of the cell line, suggesting that expression of receptors for *C. difficile* on epithelial cells increased with their differentiation. *C. difficile* adhered to HT29-MTX cells in a more diffused manner and binding to undifferentiated cells was not observed emphasising the effect of the state of differentiation of the host cells on the process. Adherent bacteria were seen to release a web of extracellular material following adhesion which promoted contact

with the cells as well as the formation of bacterial clusters. Trypsinisation destroyed adherence suggesting that the adhesins were proteinaceous in nature and two proteins were identified as putative adhesins. Further, a heat-shock between 50°C and 60°C and growth in the presence of blood increased adherence. It was thus suggested that the presence of blood in the intestinal tract could aid attachment and colonisation, although the role of heat-shock *in vivo* was debated. In another study it was observed that a heat-shock at 60°C for 10 minutes increased the adherence of *C. difficile* to Caco-2 cells but the presence of blood in the growth medium had no effect on binding (Karjalainen *et al.*, 1994). Adhesion to Vero and HeLa cells was also seen; adhesion to Vero cells was the most uniform. Heat-shocked *C. difficile* cells also attached to mucus from axenic mice *ex vivo*.

A comparison of toxigenic and non-toxigenic *C. difficile* strains showed that both adhered similarly to biopsies of colonic epithelium and small intestine epithelium *ex vivo* and also to Caco-2 and HT29 cells (Drudy *et al.*, 2001). Adherence to primary human cells was always much greater than that to the cell lines but there was considerable variability between binding to the primary cells obtained from different patients. Thus, patient factors such as genetics or environmental conditions *in vivo* could affect expression of *C. difficile* intestinal receptors and influence the outcome of disease. Also, as infection with *C. difficile in vivo* is normally restricted to the colon but the bacterium can attach to other parts of the intestine, it was hypothesised that the small intestine could serve as a reservoir for bacteria. Further, *C. difficile* toxins are not required for adherence but could facilitate binding and mucosal damage could expose more receptors (Drudy *et al.*, 2001; Waligora *et al.*, 1999). There appears to be host-strain specificity in the binding of *C. difficile*, but there is no association with toxin production (Taha *et al.*, 2007) and thus, the association between adherence and virulence remains hidden. The presence of flagella was found to be important in adherence to caecal cells in mice *in vivo* but did not affect colonisation; non-flagellated and flagellated strains colonised mice rapidly and to the same extent (Tasteyre *et al.*, 2001a).

Adherence of *C. difficile* to epithelial cells can vary with environmental factors (Hennequin *et al.*, 2001a; Waligora *et al.*, 1999). It has been observed that the adhesins are up-regulated under stresses such as partially aerobic conditions, iron-deficiency, high calcium concentrations, acidic conditions and the presence of antibiotics such as ampicillin. Further, adherence was found to be dependent on the growth-phase of the cells and the osmolarity of the medium they were grown in; maximum binding was observed in the stationary phase of growth when cells were grown in a medium with high osmolarity. These stresses could alter the bacterial surface directly or lead to the increased transcription of adherence-related genes. Adherence also varies with the state of differentiation of the host cells as mentioned above. Interestingly, three putative adhesins appeared to be constitutively expressed irrespective of environmental conditions (Waligora *et al.*, 1999) suggesting that *C. difficile* can adhere to the GI tract under normal conditions but alteration of the gut environment could enhance this process.

The aim of this study was to understand the interactions of *C. difficile* cells and their antigens with epithelial and immune cells. Five *C. difficile* strains were used as in the other studies: strain 630, strain VPI 10463 and ribotypes 027, 001 and 106. Specifically, the first aim was to measure the levels of different cytokines produced by macrophages challenged with surface antigens and toxins from these strains. The second aim was to investigate adherence of these strains to epithelial cells and determine the degree to which flagella and SLPs contribute to attachment.

5.2. Methods

Four antigens were extracted from the five *C. difficile* strains: SLPs (2.4.4), flagella (2.4.5) and crude heat-shock proteins, GroEL and Cwp66 (2.4.6). The purity of SLPs and flagella was checked by SDS-PAGE and western blotting using rabbit serum against whole *C. difficile* cells at 1:100 dilution (2.5.2) and also by LAL assay (2.4.9) and silver staining (2.4.10). Protein concentrations were determined by Bradford assay (2.4.8). Culture supernatants used were collected at 8, 12, 20 and 24 h and

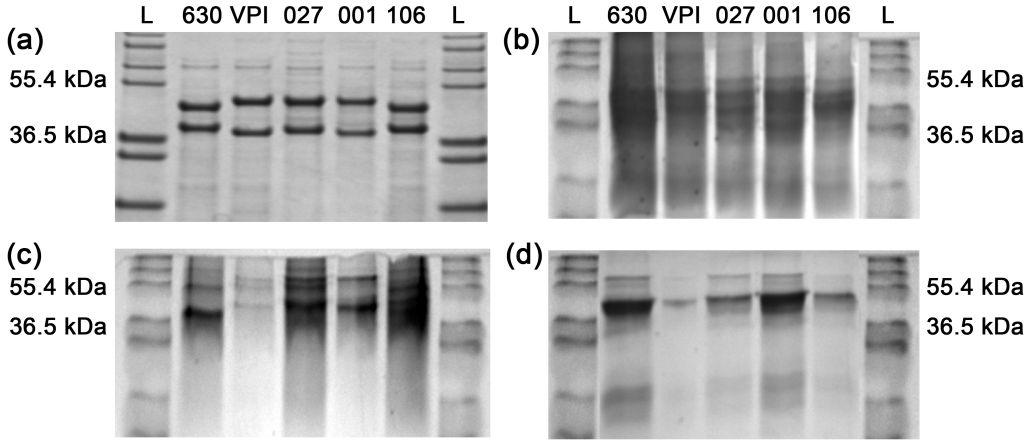
concentrations of toxins in them were determined during the experiments described in Chapter 3 (3.3.5). Genetic differences between the strains were confirmed by PCR; *slpA* (2.3.11), *fliC* and *fliD* (2.3.5) were amplified and RFLP was performed for the flagella genes. Surface-protein and toxin preparations were used for assays with THP-1 cells. The THP-1 monocyte cell line was maintained as described in 2.6.1 and 2.6.3. For the assays, cells were counted (2.6.2) and diluted appropriately before differentiation with PMA (2.6.5). This differentiation of monocytes to macrophages was confirmed visually under a microscope and by flow cytometry (2.6.6). The cell line was also checked for mycoplasma contamination (2.6.4). The macrophages were challenged with the antigens and the supernatants were collected for cytokine measurement (2.6.5). To measure cytokine production, ELISAs were developed for TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70 (2.5.6) and performed in duplicate for each antigen-challenge experiment (2.5.4). The data were analysed by non-linear regression (2.10). To study the attachment of the five *C. difficile* strains to epithelial cells, adherence assays and adherence-inhibition assays with SLPs and flagella were carried out (2.6.8).

5.3. Results

5.3.1. Preparation of *C. difficile* antigens

SLPs, flagella and heat-shock proteins at 42°C and 60°C were extracted successfully from the five *C. difficile* strains (Fig. 5.6). In the SLP extracts, two major bands were observed for the HMW SLP and the LMW SLP. The molecular weights of these proteins were determined and S-layer types 5138, 5435, 5438, 5436 and 5037 were assigned to strain 630, VPI 10463 and ribotypes 027, 001 and 106, respectively. In the flagella preparations, a prominent 39 kDa band was observed which was also the only band detected in the western blot. The blot could not be clearly photographed and hence, is not shown. A 58 kDa band was observed in the GroEL preparation as expected. In the Cwp66 preparation, three bands of approximately 66 kDa, 50 kDa and 35 kDa were observed in keeping with previous studies. All the protein samples were diluted to a concentration of 100 μg/ml.

Fig. 5.6. Protein preparations from five *C. difficile* strains

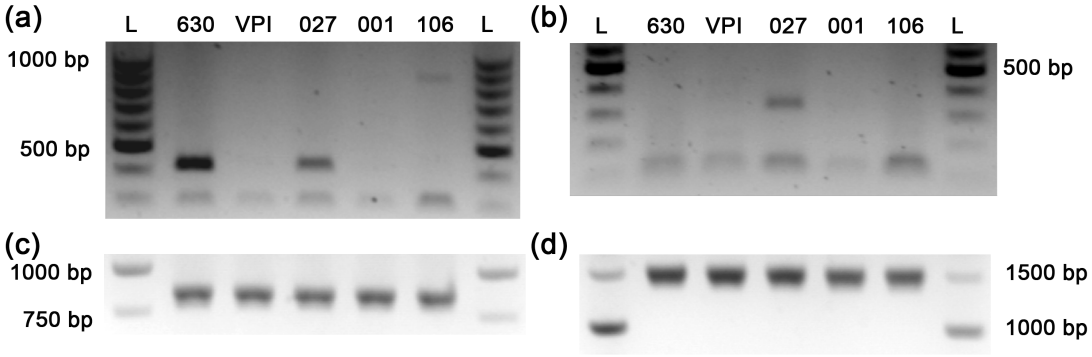


(a) SLPs, (b) flagella and crude lysates of heat-shock proteins expressed at (c) 42°C and (d) 60°C were prepared from the five *C. difficile* strains. The SLP-types for strain 630, VPI 10463 and ribotypes 027, 001 and 106 were 5138, 5435, 5438, 5436 and 5037, respectively. The 39 kDa band observed in the flagella preparations was the most prominent band, even though other bands were seen. For GroEL, a 58 kDa band was observed. For Cwp66, bands of approximately 66 kDa, 50 kDa and 35 kDa were observed.

5.3.2. Inter-strain differences in SLPs and flagella

Genes for the SLPs and flagella, *slpA*, *fliC* and *fliD*, were amplified from the five strains (Fig. 5.7).

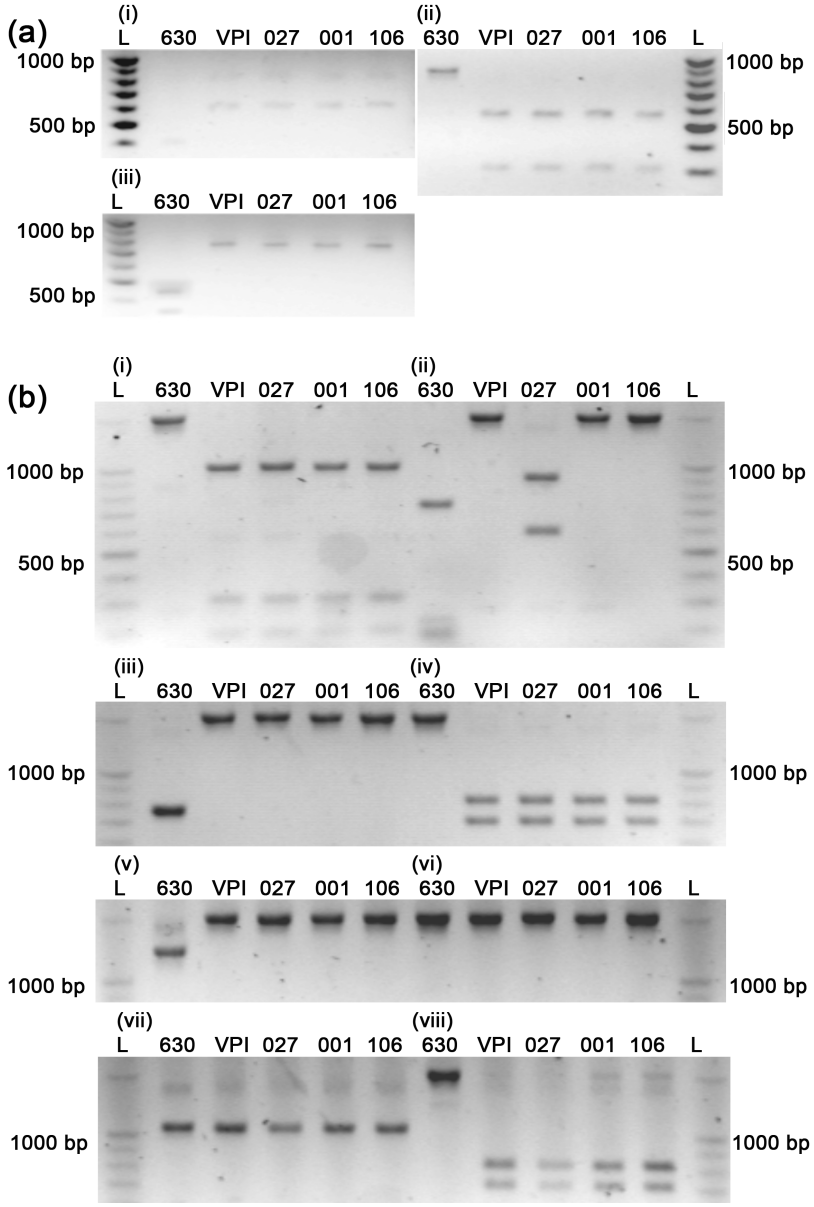
Fig. 5.7. PCR amplification of *slpA*, *fliC* and *fliD* from the five *C. difficile* strains



(a) The entire *slpA* gene and (b) its variable region were amplified by PCR. Although the products obtained were not of the expected size, it was evident that there was variability in the *slpA* gene between the *C. difficile* strains. (c) A band of 870 bp for *fliC* and (d) a 1524 bp band for *fliD* were obtained in all the strains.

PCR amplification of the entire *slpA* gene and its variable region both showed the presence of unexpected bands (Fig. 5.6.a and 5.6.b). This showed strain-specific variations in SLPs at the genetic level. PCR amplification of the flagella genes *fliC* and *fliD* gave products of 870 bp and 1524 bp, respectively (Fig. 5.6.c and 5.6.d). RFLP analysis showed that both the flagella genes were variable (Fig. 5.8).

Fig. 5.8. RFLP analysis of *fliC* and *fliD* amplified from five *C. difficile* strains

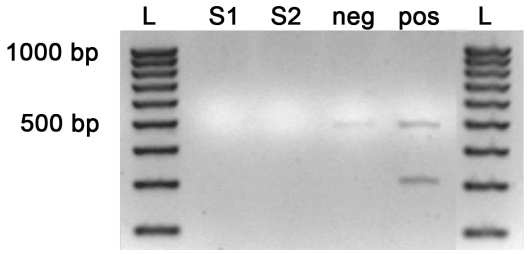


(a) *fliC* PCR products were digested with (i) *Rsa*I, (ii) *Hin*dIII and (iii) *Hpa*I (b) *fliD* products were digested with (i) *Acc*I, (ii) *Dra*I, (iii) *Eco*RI, (iv) *Hin*cII, (v) *Hin*dIII, (vi) *Hpa*I, (vii) *Rsa*I and (viii) *Xba*I. Two RFLP patterns were obtained for *fliC* and three for *fliD*.

5.3.3. Differentiation of THP-1 cells

Monocytic THP-1 cells were cultured successfully. The cell line was found to be free from mycoplasma contamination (Fig. 5.9).

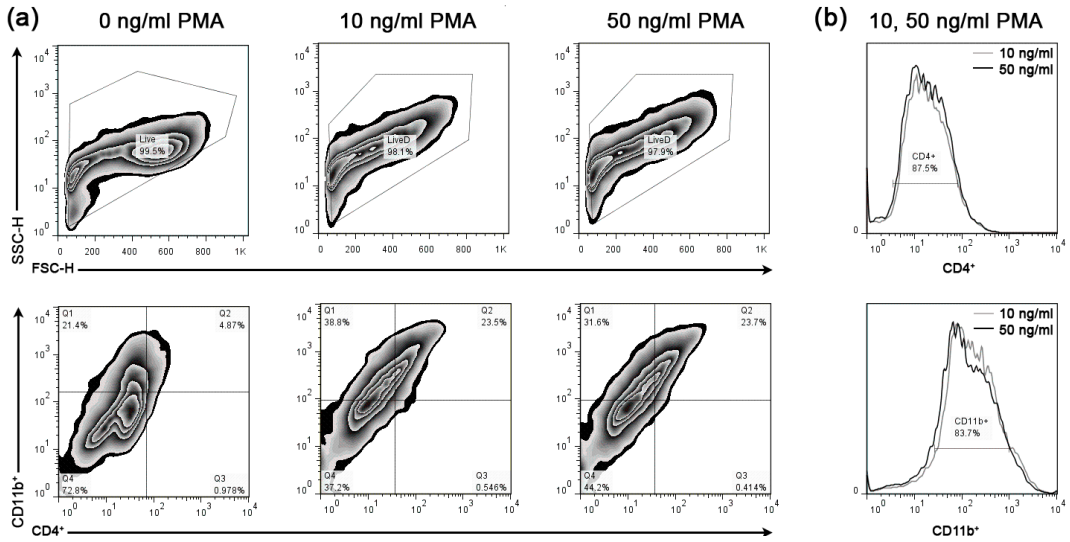
Fig. 5.9. Mycoplasma detection in THP-1 cells



The absence of a 315 bp PCR product confirmed that there was no mycoplasma contamination in the THP-1 cell line.

The THP-1 cells were differentiated with phorbol,12-myristate,13-acetate (PMA). Initially, PMA concentrations of 5, 10, 50 and 100 ng/ml were tested. PMA at 5 ng/ml was insufficient to induce differentiation, while 100 ng/ml was too high. PMA at 10 and 50 ng/ml concentrations were selected. Differentiation was confirmed by the increased expression of CD11b on the surface of the macrophages (Fig. 5.10).

Fig. 5.10. Differentiation of THP-1 cells with PMA

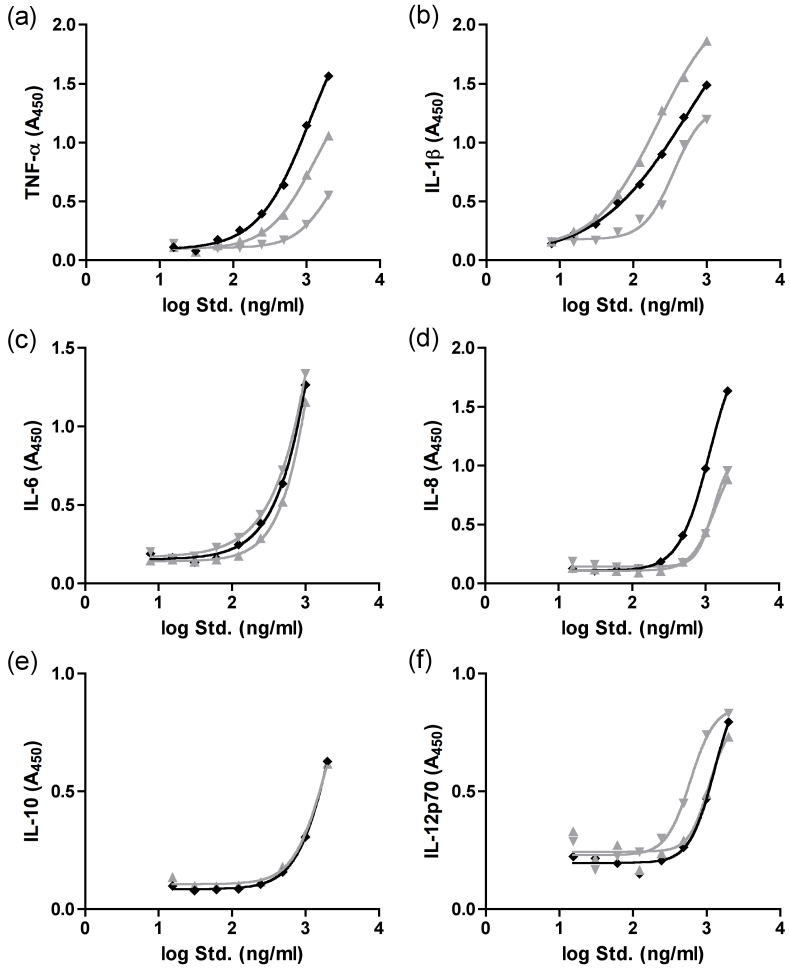


(a) PMA was used to differentiate the THP-1 monocytes into macrophages. This was confirmed by observing an increase in CD11b expression on the cells using flow cytometry. (b) PMA at 10 and 50 ng/ml generated equivalent expression of CD11b on the macrophages.

5.3.4. Development of cytokine ELISAs

In order to measure the production of cytokines by THP-1 macrophages following challenge with *C. difficile* antigens, in-house ELISAs were developed for TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70. Checkerboard assays were performed to determine suitable concentrations of the primary and secondary antibodies to be used in the ELISAs based on concentrations suggested by the manufacturers. From the standard curves generated, suitable concentration pairs were identified (Fig. 5.11).

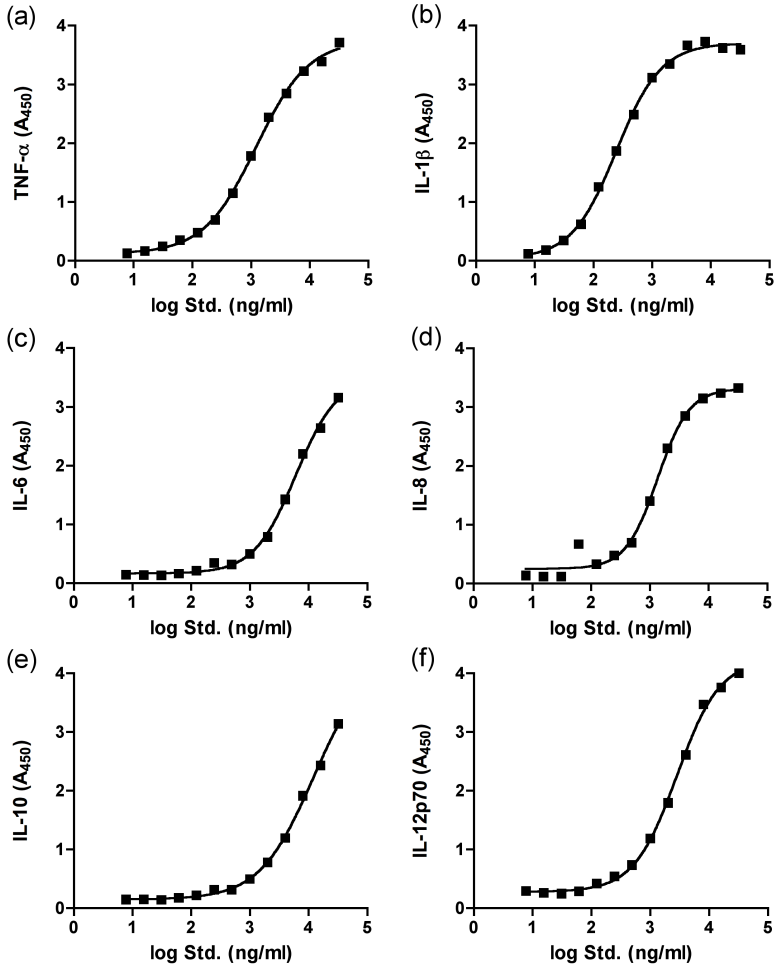
Fig. 5.11. Development of cytokine ELISAs



Checkerboard assays were performed to develop ELISAs for (a) TNF-α, (b) IL-1β, (c) IL-6, (d) IL-8, (e) IL-10 and (f) IL-12p70. Standard curves were generated for each combination of antibody concentrations used (grey). From these, the most suitable curve (black) was used to identify the appropriate concentrations of both antibodies in the final assays.

The selected antibody concentrations were tested over a wider range of protein concentrations. The experiments were performed in triplicate. The results obtained were reproducible. The standard curves showed that the ELISAs for all six cytokines were suitable to detect cytokines in the range of 32 ng/ml up to a minimum concentration of at least 31.25 pg/ml (Fig. 5.12). The antibodies used in these ELISAs, their manufacturers and the concentrations at which they were used are listed in Table 5.1.

Fig. 5.12. Standardisation of cytokine ELISAs



ELISAs for (a) TNF-α, (b) IL-1β, (c) IL-6, (d) IL-8, (e) IL-10 and (f) IL-12p70 were standardised and were found to be sensitive and reproducible.

Table 5.1. Antibodies used in cytokine ELISAs and their working concentrations

Cytokine	Primary antibody and concentration	Standard and starting concentration	Secondary antibody and concentration
TNF-α	Affinity Purified anti-human TNF-α (eBioscience, 14-7348; clone MAb1) 2 µg/ml	Recombinant Human TNF-α (PeproTech, 300-01A) 32 ng/ml	Biotin anti-human TNF-α (eBioscience, 13-7349; clone MAb11) 2 µg/ml
IL-1β	Affinity Purified anti-human IL-1β (eBioscience, 14-7018; clone CRM56) 2 µg/ml	Recombinant Human IL-1β (PeproTech, 200-01B) 32 ng/ml	Biotin anti-human IL-1β (eBioscience, 13-7016; clone CRM57) 0.25 µg/ml
IL-6	Affinity Purified anti-human IL-6 (eBioscience, 14-7069; clone MQ2-13A5) 2 µg/ml	Recombinant Human IL-6 (PeproTech, 200-06) 32 ng/ml	Biotin anti-human IL-6 (eBioscience, 13-7068; clone MQ2-39C3) 1 µg/ml
IL-8	Affinity Purified anti-human IL-8 (PeproTech, 500-M08) 3 µg/ml	Recombinant Human IL-8 (eBioscience, 14-8089) 32 ng/ml	Biotin anti-human IL-8 (PeproTech, 500-P2Bt) 0.5 µg/ml
IL-10	Affinity Purified anti-human IL-10 (eBioscience, 14-7108; clone JES3-9D7) 2 µg/ml	Recombinant Human IL-10 (PeproTech, 200-10) 32 ng/ml	Biotin anti-human IL-10 (eBioscience, 13-7109; clone JES3-12G8) 1 µg/ml
IL-12p70	Affinity Purified anti-human IL-12 (eBioscience, 14-7128; clone B-T21) 2 µg/ml	Recombinant Human IL-12 (PeproTech, 200-12) 32 ng/ml	Biotin anti-human IL-12 (eBioscience, 13-7129; clone C8.6) 1 µg/ml

5.3.5. Cytokine response to *C. difficile* antigens

THP-1 macrophages were challenged with the various *C. difficile* antigens to study the immune response elicited by them. Macrophages were incubated with SLPs, flagella and heat-shock proteins of the five strains individually at concentrations of 50, 25 and 5 µg/ml. Supernatants were collected at 4 h and 24 h and the amounts of the various cytokines present in them were tested using the ELISAs developed. In the experiments with culture supernatants, the challenge lasted only 3 h following which

the macrophages were washed and fresh medium was added to them. This was done to prevent extensive cytotoxicity due to potentially high concentrations of the *C. difficile* toxins in the supernatants. The levels of toxin A and toxin B in the culture supernatants of the five strains are listed in Table 5.2. Supernatants for cytokine measurements were collected 4 h and 24 h after this.

Table 5.2. Average concentrations of toxins in culture supernatants of five *C. difficile* strains as determined by ELISA and cytotoxicity assay

	Time (h)	Strain 630	Strain VPI 10463	Ribotype 027	Ribotype 001	Ribotype 106
Toxin A (ng/ml)	8	10.1	11.7	49.1	9.8	14.8
	12	10.2	28.1	477.2	14.6	24.8
	20	46.3	1234.2	1298.0	99.2	121.0
	24	71.3	1522.0	1674.6	177.8	317.5
Toxin B (ng/ml)	8	95.6	128.9	874.6	57.7	35.7
	12	91.2	195.2	844.9	73.6	34.2
	20	70.7	876.2	1490.4	141.2	83.8
	24	54.9	1353.9	1117.8	250.7	285.7

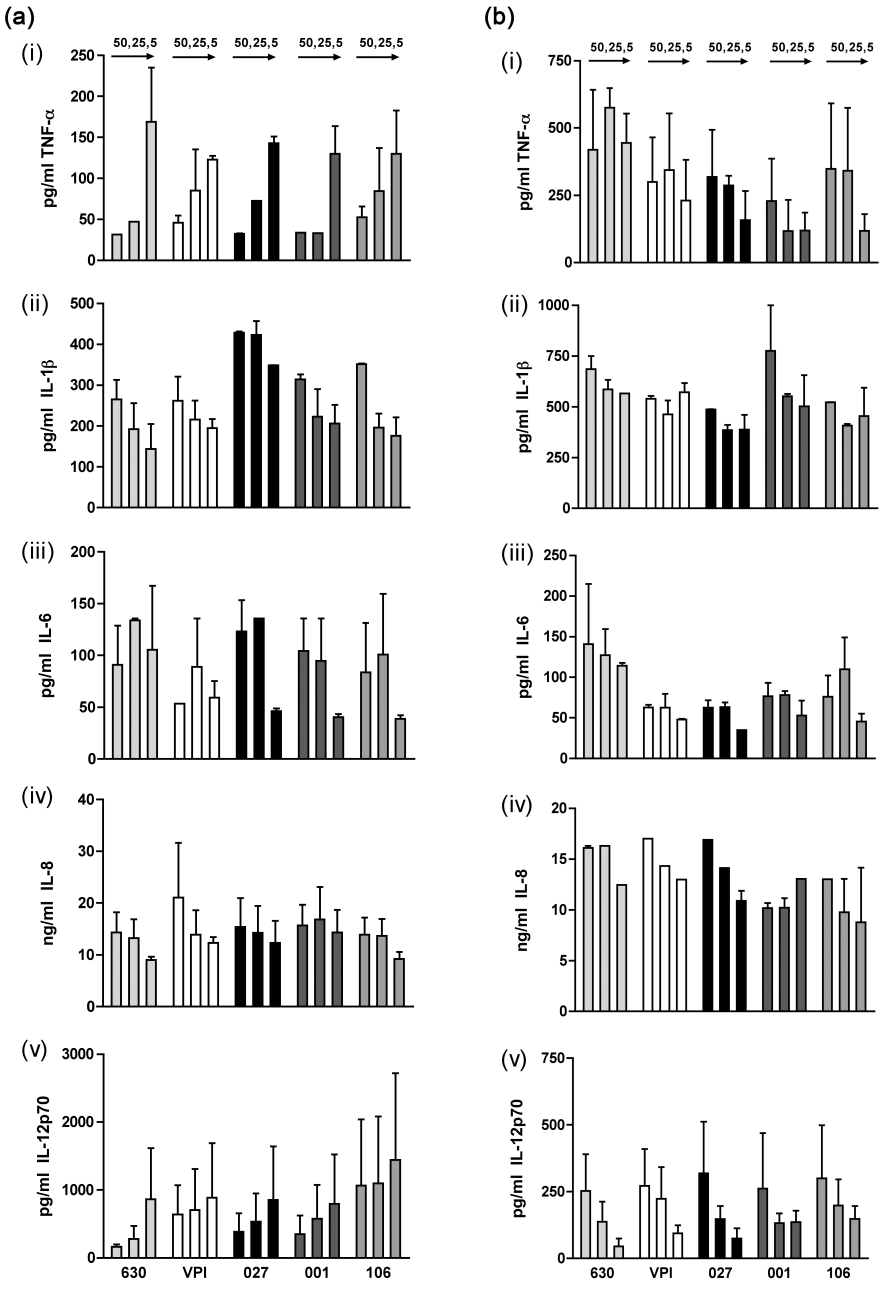
THP-1 cells differentiated with 10 ng/ml PMA and 50 ng/ml PMA were used simultaneously in the experiments. In preliminary studies, it was observed that even though there was no obvious difference between the two treatments with respect to morphological alterations of the monocytes into macrophages or the increase in expression of CD11b in the differentiated cells, there was a marked difference in the levels of cytokines released. Levels of IL-1β and IL-8 released were significantly higher in macrophages differentiated with 10 ng/ml PMA as compared to those differentiated with 50 ng/ml PMA. Also, a dose response for these cytokines was evident with dilutions of the antigens only when using macrophages differentiated with 10 ng/ml PMA and not 50 ng/ml PMA. This could be due to large amounts of cytokine being produced initially and leading to toxicity. For TNF-α, IL-6, IL-10 and

IL-12p70, macrophages differentiated with 10 ng/ml PMA produced low levels of cytokines irrespective of the concentration of antigen. When cells differentiated with 50 ng/ml PMA were used there was a marked increase in the levels of cytokines as well as a notable difference in the response elicited by different antigen concentrations. Thus, the results presented here for the cytokines are taken from the experimental setting in which optimum dose responses were detected.

The SLPs (5.13.a), flagella (5.13.b) and crude GroEL (5.14.a) and Cwp66 preparations (5.14.b) of the five *C. difficile* strains under consideration all elicited a pro-inflammatory response from macrophages. TNF-α, IL-1β, IL-6, IL-8 and IL-12p70 were produced by the macrophages. IL-10 production was not detected. IL-8 was the most abundantly produced cytokine and all the antigens induced similar levels of IL-8 production. The amounts of IL-1β and IL-6 produced in response to the antigens were similar. The amount of IL-12p70 released by macrophages stimulated with SLPs was the highest. For IL-12p70 production in response to SLPs and Cwp66, a negative dose response was observed possibly due to toxicity resulting from high antigen concentrations. Similar results were obtained for TNF-α with these two antigens. Cwp66 seemed to induce the most production of TNF-α, with flagella and GroEL inducing intermediate levels and SLPs inducing the lowest levels of TNF-α production. A negative dose response was also observed for IL-6 production by macrophages challenged with Cwp66. No significant differences were identified in the immune response generated against antigens of historic or epidemic isolates.

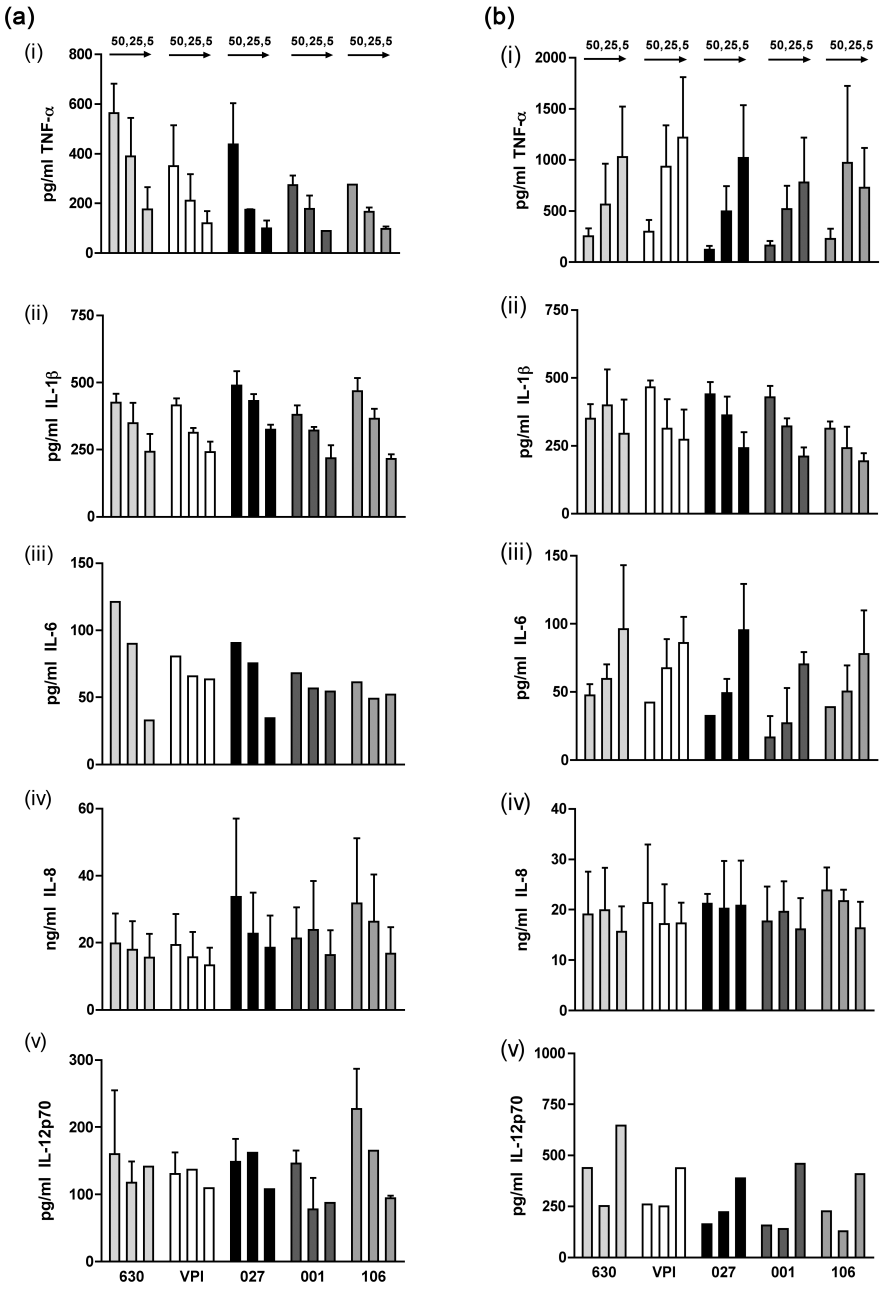
Culture supernatants of the five *C. difficile* strains induced the production of TNF-α, IL-1β and IL-8 (Fig. 5.15). There was an increase in TNF-α production in the late stationary phase of growth (20 and 24 h) as compared to the late exponential phase (8 and 12 h) which correlated with the levels of toxin A and toxin B in the culture supernatants. A more gradual increase in IL-1β levels was observed. IL-8 production was similar for all the samples but toxicity due to high levels of IL-8 was indicated. No significant differences were identified between historic, endemic and hypervirulent strains.

Fig. 5.13. Cytokine response by THP-1 macrophages to SLPs and flagella extracted from five *C. difficile* strains



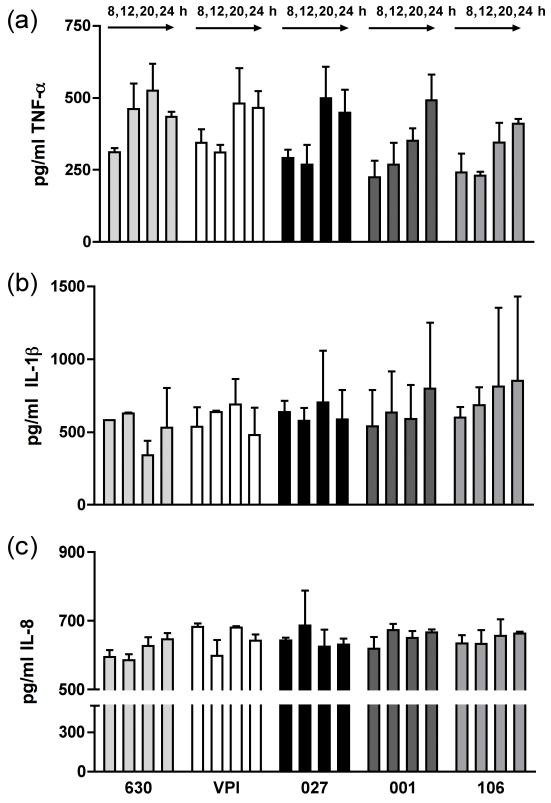
(a) SLPs and (b) flagella of the five *C. difficile* strains both induced the production of pro-inflammatory cytokines by macrophages. The levels of TNF-α and IL-12p70 produced in response to the antigens differed considerably. On challenge with SLPs, negative dose responses were observed for these cytokines, suggesting that they could be immunogenic even in small amounts. No inter-strain differences were identified. Bars indicate +/- SEM of 2 experiments.

Fig. 5.14. Cytokine response by THP-1 macrophages to heat-shock proteins released by five *C. difficile* strains at 42°C and 60°C



Crude preparations of heat-shock proteins (a) GroEL and (b) Cwp66 released by the five *C. difficile* strains induced the production of pro-inflammatory cytokines by macrophages. The amounts of cytokine, except TNF-α, detected in response to these antigens were similar. Negative dose responses were observed for TNF-α, IL-6 and IL-12p70 with Cwp66, indicating that even low amounts of the protein were sufficient to elicit an immune response. No inter-strain differences were identified. Bars indicate +/- SEM of 2 experiments.

Fig. 5.15. Cytokine response by THP-1 macrophages to culture supernatants of five *C. difficile* strains

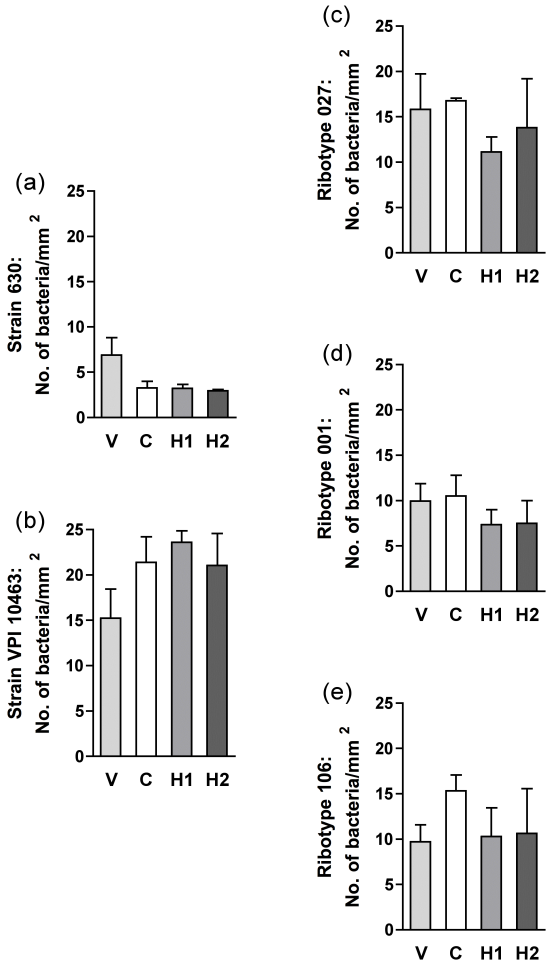


(a) TNF-α production was greater in response to culture supernatants collected in the stationary phase of growth (20, 24 h) as compared to the exponential phase (8, 12 h), corresponding with the greater amounts of extracellular toxin detected in the stationary phase. (b) IL-1β levels were also higher in response to the stationary phase samples, although the increase was less prominent. (c) The amount of IL-8 produced remained low and did not vary between the samples and toxicity of the macrophages was suspected. Inter-strain differences were not identified. Bars indicate +/- SEM of 2 experiments.

5.3.6. Adherence of *C. difficile* to epithelial cells

Adherence of exponential phase cultures of the five *C. difficile* strains to different types of epithelial cells was investigated. The four cell types used were Vero cells, Caco-2 cells and non-mucus secreting and mucus-secreting HT29 cells. Adherence of all the strains to all the cell lines tested was observed (Fig. 5.16). The presence of mucus was not found to increase attachment of bacterial cells. The extent of binding observed was not dependent on the cell line but on the strain of *C. difficile*. Interestingly, VPI 10463 showed maximum adherence to all the cell lines, while strain 630 was the least adherent strain. Of the epidemic isolates, ribotype 027 exhibited the greatest ability to bind to epithelial cells, followed by ribotypes 106 and 001, respectively.

Fig. 5.16. Adherence of five *C. difficile* strains to epithelial cells



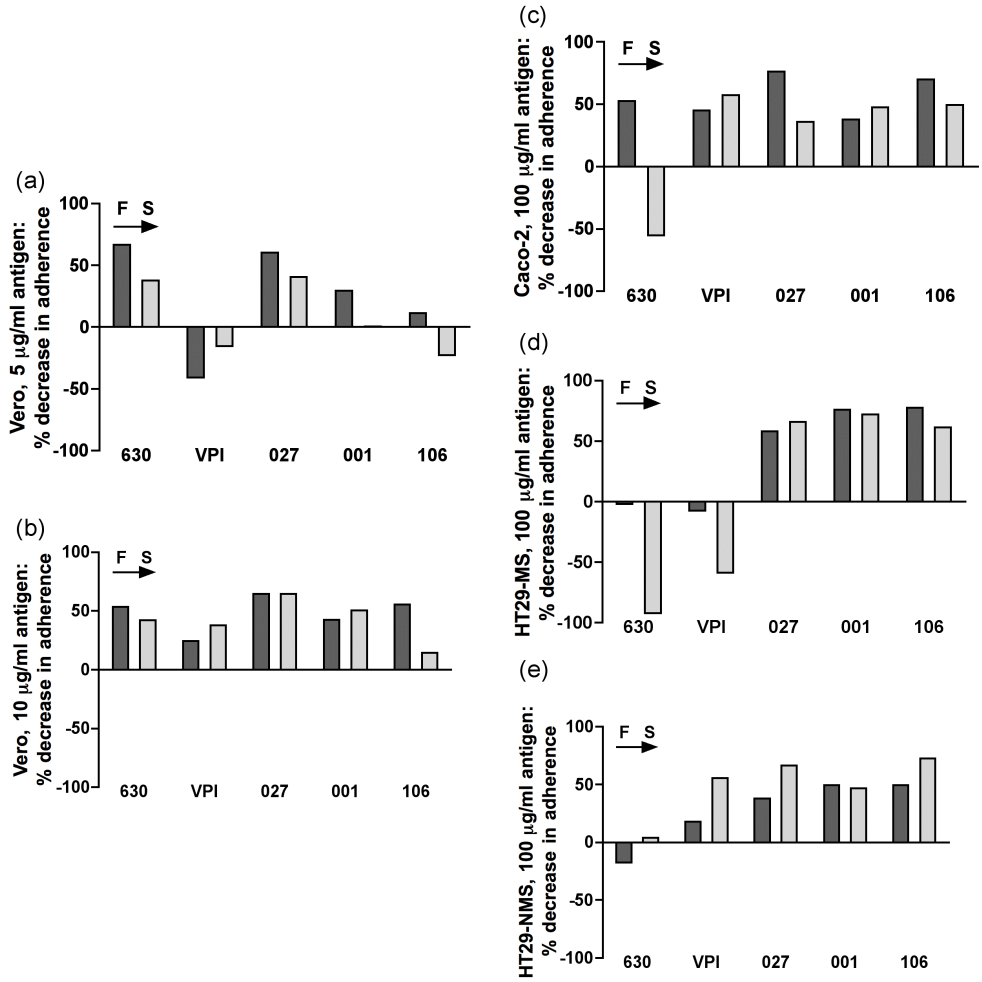
Attachment of *C. difficile* cells to epithelial cells in the exponential phase of growth was investigated. Four cell lines were used: Vero (V), Caco-2 (C), HT29-NMS (H1) and HT29-MS (H2). The numbers of bacterial cells attached to epithelial monolayers on the cover slips were enumerated microscopically. (a) Strain 630 showed the least attachment, while (b) strain VPI 10463 showed the most. Of the epidemic strains, (c) ribotype 027 was the most adherent, (d) ribotype 001 was the least and (e) ribotype 106 exhibited intermediate attachment. The degree of adherence was found to be independent of the type of cell line used. Bars indicate +/- SEM of 2 experiments with Caco-2 and HT29-MS cells and 4 experiments with Vero and HT26-NMS cell lines.

5.3.7. Role of SLPs and flagella in adherence

Adherence-inhibition assays were performed to study the extent to which SLPs and flagella contribute to the attachment of *C. difficile* to epithelial cells (Fig. 5.17). Preliminary assays were performed with Vero cells only due to the ease of culture of this cell line. Vero cells were pre-incubated with SLPs and flagella before addition of exponential phase cultures of the five *C. difficile* strains. Proteins were tested for inhibition of attachment of only the strains from which they were prepared. Proteins were originally tested at concentrations of 5 μg/ml and 10 μg/ml. At 5 μg/ml, marked inhibition of binding by both proteins was observed only for strain 630 and ribotype 027. At 10 μg/ml, both proteins caused notable inhibition of adherence and at similar levels, except in ribotype 106, in which flagella were more inhibitory than SLPs.

With Caco-2 and HT-29 cells, 100 μg/ml antigens were tested. Inhibition of binding to Caco-2 and HT29-NMS cells by flagella and SLPs was seen in all the strains. With HT29-MS cells, inhibition was only observed for the epidemic strains; increased binding and aggregation of bacteria were detected for strains 630 and VPI 10463.

Fig. 5.17. Role of flagella and SLPs of five *C. difficile* strains in adherence to epithelial cells



Adherence-inhibition by flagella (dark grey) and SLPs (light grey) of the *C. difficile* strains was demonstrated. Vero cells were treated with (a) 5 and (b) 10 μg/ml of proteins and reduced adherence was observed with the latter. With the other cell lines, proteins were tested at 100 μg/ml. (c) Decreased adherence to Caco-2 cells was observed with the strains with flagella, but not SLPs. (d) Using HT29-MS cells, flagella and SLPs decreased attachment of only the epidemic ribotypes. (e) With HT29-NMS cells, only the flagella of strain 630 did not decrease adherence; inhibition was observed in the other strains.

5.4. Discussion

Mucosal surfaces are the main sites of host-pathogen interactions (Ramos *et al.*, 2004). Pathogenic bacteria express a wide range of surface organelles and secrete virulence factors such as toxins which directly mediate these interactions. From the results of this study, it is evident that the virulence factors of *C. difficile* can interact in a dynamic way with human epithelial and immune cells during an infection.

The virulence factors studied here are all recognised by the immune system of patients with CDI and even carriers as determined by antibody detection in their sera (Péchiné *et al.*, 2005a; Wright *et al.*, 2008). However, apart from the toxins (Flegel *et al.*, 1991; Linevsky *et al.*, 1997; Melo Filho *et al.*, 1997) very little is known about the interactions of the other virulence factors with cells of the innate immune system. In keeping with previous observations, it was found that culture supernatants collected during the growth of five *C. difficile* strains were able to elicit a strong pro-inflammatory response; the production of TNF-α, IL-1β and IL-8 was detected (Canny *et al.*, 2006). Late exponential phase supernatants were slightly less immunogenic than late stationary phase supernatants. Although these observations correlated well with the amounts of toxin A and toxin B present in the supernatants, the presence of other cell wall components such as lipoteichoic acid cannot be ruled out. Interestingly, even though culture supernatants of *C. difficile* ribotype 027 and strain VPI 10364 contained approximately ten times more toxin than those of the other strains, the level of cytokine production by the supernatants of all the strains was not significantly different. It is possible that the large amounts of toxin rapidly induced toxicity in the macrophages even after exposure for three hours.

It has been observed previously that exposure of monocytes to toxin B was lethal; 500 ng toxin B was lethal and even 5 ng of toxin B resulted in the death of 75% of monocytes within just five hours (Flegel *et al.*, 1991). Further, macrophages were found to be more sensitive to the toxic effects of *C. difficile* toxins than monocytes (Linevsky *et al.*, 1997) suggesting that greater and more rapid cell death could have occurred during the toxin-shock. It has been suggested that the release of pro-

inflammatory cytokines followed by cell death could render monocytes unable to carry out phagocytosis, which could foster inflammation (Flegel *et al.*, 1991). It was also curious to detect rather low levels of IL-8 production with all the supernatants, especially when compared to the amounts produced in response to surface-associated proteins. This observation suggested that a toxic environment was generated either directly by the toxins themselves or by the large amounts of cytokines being produced in response to them.

The four cell surface-associated proteins extracted from the five *C. difficile* strains were also found to induce the production of pro-inflammatory cytokines by macrophages. S-layer proteins (SLPs) have been shown to be strongly immunogenic (Ausiello *et al.*, 2006). Monocytes challenged with SLPs from strain 630 were found to induce the production of large amounts of IL-1β and IL-6 pro-inflammatory cytokines. They also induced maturation in monocyte-derived dendritic cells, altering their function from antigen-processing to antigen-presenting cells and increased proliferation of allogenic T cells. They were found to be capable of driving both IL-12p70-mediated Th1 and IL-10-mediated Th2 responses, thus affecting antibody responses. Similar results were observed in another study comparing SLPs from different *C. difficile* strains (Bianco *et al.*, 2011). SLPs of hypervirulent epidemic and non-hypervirulent, non-epidemic strains induced production of similar levels of IL-1β, IL-6 and IL-10. IL-12p70 production in response to SLPs of all the strains was negligible, except those of strain 630, which induced considerable production of IL-12p70. These two studies showed that *C. difficile* SLPs have an important role in the immune response generated in CDI.

In the study presented here, SLPs of the five *C. difficile* strains, which include three of the strains used in the above-mentioned studies, were found to induce only pro-inflammatory cytokines; IL-10 production was not detected. Although the amount of protein used in the assay and the time of cytokine detection were similar, it is possible that the differences lie in the immune cells used in both studies. Monocytes purified from peripheral blood mononuclear cells were used in the published studies,

while THP-1 macrophages were used here. However, the potential of SLPs as immunogens and the lack of correlation of the immune response to the SLPs with strain-type were clearly observed.

Flagella of the five strains also induced pro-inflammatory cytokine production at equivalent levels. Most investigations of flagella have been performed in Gram-negative organisms. Flagella have been found to stimulate the production of the pro-inflammatory cytokines TNF-α and IL-6 even at low concentrations but they have also been found to induce Th2 responses and there appears to be an association between the dose of flagellin and the type of response induced (Ramos *et al.*, 2004). Interactions of flagella with epithelial cells are perhaps the most important in infection. Flagella of *Salmonella enterica* can stimulate IL-8 production by epithelial cells and also induce the production of factors such as nitric oxide, chemokines and defensins that are involved in the recruitment of inflammatory cells (Ramos *et al.*, 2004; Viswanathan *et al.*, 2004). Filamentous flagella do not induce pro-inflammatory responses, possibly owing to the exposure of only its conserved domains. However, flagellin monomers induce pro-inflammatory cytokines. It is likely that the delivery of monomers as part of pathogenesis or degradation of flagella by host proteases results in inflammation. Thus, the type of flagellar protein presented to the immune system could alter the outcome of infection.

Heat-shock proteins (HSPs) of *C. difficile* were also highly pro-inflammatory and it can be suggested that Cwp66 was more potent than GroEL as significant production of TNF-α and IL-6 was observed even with the highest dilution of protein used. Since these preparations were also crude extracts, the contribution of other proteins and lipocarbohydrate to the cytokine response cannot be discounted. Also, in the experiments with HSPs, no inter-strain differences were identified. This is perhaps not surprising as heat-shock proteins are the most highly conserved proteins in the biosphere (Zügel & Kaufmann, 1999). HSPs accumulate rapidly under stressful conditions in many organisms and this has also been demonstrated in *C. difficile* (Hennequin *et al.*, 2001a; Waligora *et al.*, 1999). They are vital for the survival of

pathogens in the host where they encounter changes in temperature and pH and even oxygen radicals, enzymes and iron-deficiency on internalisation into macrophages (Zügel & Kaufmann, 1999). They can also be highly immunogenic because of their abundance under stressful conditions and also due to cross-reactive memory owing to their highly conserved nature.

The data presented here identified four *C. difficile* surface-associated proteins as possible mediators of the inflammation observed in CDI, along with the toxins. The level of cytokine production in response to the proteins was independent of the strain used. Thus, surface-proteins do not contribute to the increased virulence observed in the currently epidemic ribotypes 027, 001 and 106. The large volumes of toxin produced by the hypervirulent ribotype 027 might elicit a greater immune response *in vivo* due to extensive damage leading to chronic inflammation but this could not be identified from the results obtained here. Although only pro-inflammatory responses were detected for the toxins and surface-associated proteins, it is likely that the SLPs and flagella are also capable of driving anti-inflammatory responses, as observed by others (Ausiello *et al.*, 2006; Bianco *et al.*, 2011). One of the limitations of this study was the limited range of dilutions over which the antigens were tested. In the cases where negative dose responses were observed, it was not possible to determine the lowest concentration of protein that may be immunogenic. Dilutions of the culture supernatants could have also indicated the minimum amount of toxin required to elicit an immune response. Also, as macrophage survival was not assessed, it was not possible to determine if the proteins were toxic to them.

The adherence of *C. difficile* to mucosal surfaces is important in the establishment of infection (Borriello *et al.*, 1988; Drudy *et al.*, 2001). Adhesins including flagella have been observed to be involved in adhesion (Hennequin *et al.*, 2001a; Tasteyre *et al.*, 2001a; Waligora *et al.*, 1999). The role of toxins in this process, however, has been debated (Borriello *et al.*, 1988; Drudy *et al.*, 2001). In this study, *C. difficile* cells were found to attach to different types of epithelial cells under anaerobic conditions. The type of cell line used and the presence or absence of mucus was not

found to affect the extent to which *C. difficile* strains attached to the cells. However, the degree of attachment depended on the strain in question. Surprisingly, strain VPI 10463, which is a high toxin producer and is seldom implicated in infection, was found to be the most adherent strain. In contrast, strain 630, which was isolated from a patient with severe disease during an outbreak (Wüst *et al.*, 1982) was the least adherent. Of the current epidemic strains, ribotype 027 showed the most binding, followed by ribotypes 106 and 001. This clearly shows that the currently epidemic strains have an advantage over the historic strain 630. Interestingly, the adherence results correlated with the pattern of toxin production by these strains in the exponential phase as described in Chapter 3 (Fig. 3.20). However, as toxin production was not assessed here and neither were the experiments performed with cells that had been separated from the toxin, this association cannot be confirmed.

The role of toxins in adherence could be determined by assessing toxin production at the time of adding *C. difficile* cells to epithelial cells and by monitoring toxin production throughout incubation. Initial amounts of toxin could be removed by washing or neutralised with anti-toxin. It has also been suggested that adherence can be influenced by the binary toxin of *C. difficile*. The binary toxin has been shown to induce the formation of long microtubule-based protrusions on the surface of epithelial cells that can form a mesh and increase the adherence of bacteria (Schwan *et al.*, 2009). The microtubules have been found to enhance the attachment of *C. difficile* to epithelial cells by approximately four-fold *in vitro* under anaerobic conditions. Of the five strains studied here, only ribotype 027 produces the binary toxin as confirmed from the results in Chapter 6 (Fig. 6.7). Therefore, increased adherence of strain VPI 10463 was not a consequence of the binary toxin. Also, from these preliminary results it can be suggested that the binary toxin did not contribute significantly to adherence.

Flagella and SLPs of the five strains were found to contribute almost equally to the adherence of the strains from which they were extracted. Inhibition of adherence was observed under most experimental conditions suggesting that the proteins directly

contributed to adhesion. Flagella are an important virulence factor and play an important role in adhesion and colonisation of several bacteria (Ramos *et al.*, 2004). The variable domain of flagella, which is believed to induce inflammatory immune responses, is also suggested to mediate adhesion. In enteric organisms such as *Vibrio cholerae*, transcription of other virulence factors is linked to flagella expression. No such association has been found in *C. difficile* as yet and although the role of flagella in attachment has been clearly demonstrated in mice, non-flagellated *C. difficile* strains were less adherent than flagellated strains (Tasteyre *et al.*, 2001a). SLPs have been shown to be responsible for adhesion in *Lactobacillus acidophilus* and some *Bacillus* species (Sára & Sleytr, 2000). They have also been seen to bind to basement membrane proteins and a role for SLPs in autoagglutination has been indicated (Sleytr & Messner, 1993). Thus, SLPs of *C. difficile* could mediate attachment not just at the initial stages of infection but also to the basement membrane once the epithelial barrier is destroyed by toxins.

Where inhibition of adherence to epithelial cells was not observed, the opposite effect was seen; instead of preventing binding of bacteria, areas of high bacterial density were observed on the monolayers. The formation of clusters by adherent *C. difficile* cells has been observed previously owing to the release of extracellular materials (Eveillard *et al.*, 1993). Although no obvious extracellular matrix was observed microscopically in this study, it is possible that this was the case and that the flagella helped form this web. *C. difficile* SLPs could cause this effect by mediating autoagglutination (Sleytr & Messner, 1993). If such interactions occurred *in vivo*, it would explain the characteristic pathology observed in *C. difficile*. Often in pseudomembranes, small ulcerations surrounded by healthy mucosa are observed (Johal *et al.*, 2004c). Bacterial clusters attached to the mucosa could result in targeted release of the toxins in small areas of the gut, which could induce massive inflammation in those areas causing ulceration.

Cross-reactions with proteins tested against strains from which they were not extracted were not performed. However, it would be interesting to know if the

presence of one strain or its antigens could inhibit colonisation by another. This would be most useful if competition between a toxigenic and a non-toxigenic strain was assessed. It would also have been interesting to investigate the role of heat-shock proteins in adherence, but this was not possible owing to limitations of time and amount of protein available. Although there is no temperature shock *in vivo*, aerobic, acidic and nutrient-shocks could result in the increased expression of large amounts of HSPs, which might contribute significantly to attachment. Moreover, as these proteins play an important role in physical attachment to the mucosa while also stimulating the immune system, they could be used as constituents of multi-component vaccines for *C. difficile*. Vaccination of hamsters and mice with SLPs has been attempted and has showed promising results (Ní Eidhin *et al.*, 2008; O'Brien *et al.*, 2005). HSPs have been used as carrier molecules in vaccines (Zügel & Kaufmann, 1999). However, intensive studies of the interactions of purified proteins in cell lines and in animal models will be required for vaccine development.

Although macrophages are crucial to the immune responses in the gut, the epithelium is the first point of contact for pathogens like *C. difficile*. This is the site of adherence as determined here and in previous studies. Moreover, cultures of *C. difficile* and their toxins have been found to induce the production of pro-inflammatory cytokines by epithelial cells (Canny *et al.*, 2006; Johal *et al.*, 2004c; Ng *et al.*, 2003; Savidge *et al.*, 2003). Thus, studying the cytokine response by the epithelium during attachment would be useful. It could indicate which proteins are more likely to induce a Th2 anti-inflammatory response and which drive a Th1 pro-inflammatory response. Canny and colleagues used a ‘sided’ system to compare apical and basolateral responses to *C. difficile* cultures. Similar experiments could be performed with purified antigens (Canny *et al.*, 2006). Further experiments assessing interactions of bacteria, epithelial cells and dendritic cells could be performed to understand the immune response in greater detail. Such experiments have been performed with *S. enterica*, in which IL-10 was produced when monocyte-derived dendritic cells were directly confronted with the bacterium or indirectly through epithelial cells, but IL-12 was produced only when there was direct contact of the dendritic cells with

bacteria (Rimoldi *et al.*, 2005a). Thus, invasion of the epithelium was required to drive a pro-inflammatory response. Although *C. difficile* is not an invasive bacterium, its toxins disrupt epithelial barrier function (Nusrat *et al.*, 2001) and increased expression of adhesion molecules for greater recruitment of neutrophils requires basolateral exposure to *C. difficile* (Canny *et al.*, 2006). The responses of epithelial and immune cells during such interaction would provide useful insights into the inflammation caused by *C. difficile in vivo*. However, studies in animal models would be most informative.

6. Hypervirulent *C. difficile*

6.1. Introduction

C. difficile was identified as the cause of antibiotic-associated pseudomembranous colitis in 1978 and has been the leading cause of nosocomial diarrhoea in the developed world ever since. However, the early 2000s witnessed a change in the epidemiology of *C. difficile* infection the world over. A new strain of *C. difficile* with increased virulence and resistance was discovered. This is the dreaded strain known today as the hypervirulent ribotype 027 (also designated as NAP1 or BI). The clonal spread of ribotype 027 was studied by comparing phenotypic and genetic characteristics of isolates collected in Scotland and the Netherlands.

6.1.1. Emergence of hypervirulent *C. difficile*

An increase in the number of patients with more severe disease and increased morbidity, mortality and relapse was first identified in Canada during the latter half of 2002 (Miller *et al.*, 2002; Pépin *et al.*, 2004; Warny *et al.*, 2005). A comparison of the trends of CDI from 1991 to 2003 in Canada revealed that incidence had increased from 35.6 per 100,000 to 156.3 per 100,000 in the Sherbrooke region of Quebec and even in areas where incidence was previously stable, a sudden rise to 92.2 per 100,000 was observed in 2003 (Pépin *et al.*, 2004; Pépin *et al.*, 2005b). This marked the beginning of a series of outbreaks in Canada and the extensive use of fluoroquinolones was identified as a major contributing factor to these (Pépin *et al.*, 2005a). A simultaneous outbreak in 12 hospitals in Quebec was observed (Loo *et al.*, 2005). Further, a survey of the different regions of Canada showed that ribotype 027 was endemic in the country and was associated with both nosocomial and community-acquired infection (MacCannell *et al.*, 2006). At about the same time in the USA, outbreaks caused by a similar strain were reported (Centers for Disease Control and Prevention, 2005; McEllistrem *et al.*, 2005; Muto *et al.*, 2005).

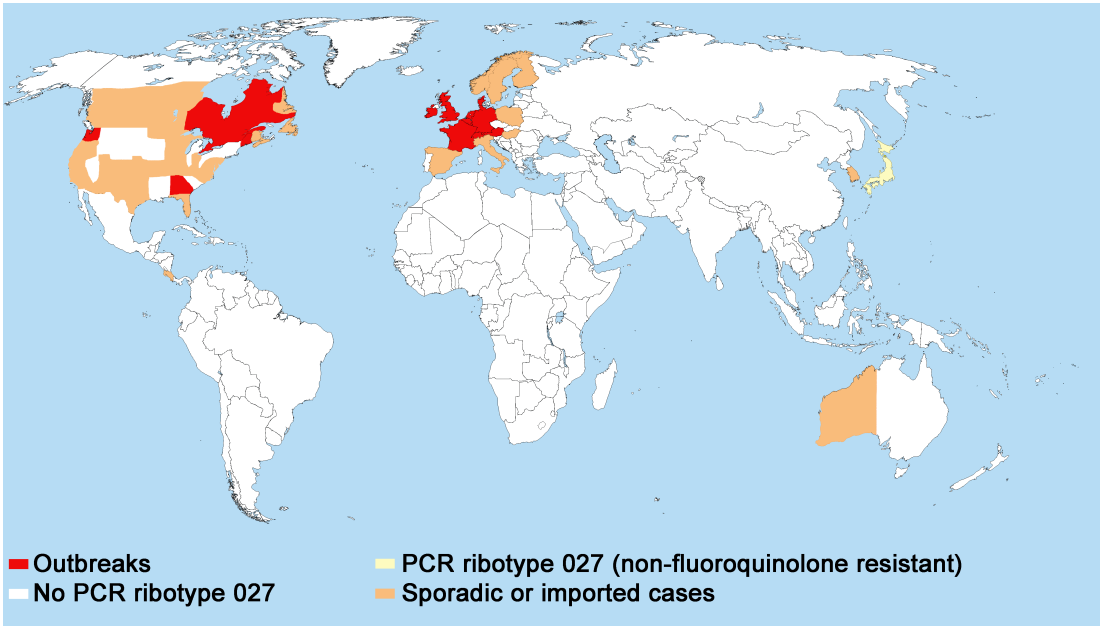
Between October 2003 and June 2004, an outbreak caused by *C. difficile* ribotype 027 was identified at the Stoke Mandeville hospital in England, followed by a second

outbreak between October 2004 and June 2005 (Healthcare Commision, 2006). Another ribotype 027 outbreak then occurred at the Royal Devon and Exeter hospital in 2005 and this was related to a change in antibiotic usage (Kuijper *et al.*, 2006). The next series of outbreaks occurred in the Netherlands in Harderwijk (van Steenbergen *et al.*, 2005); three clusters of CDI were identified, the third being unrelated to the first two. Amsterdam and other parts of the country also witnessed ribotype 027 outbreaks (Kuijper *et al.*, 2008). In the surveillance period from 2005 to 2007, sixteen outbreaks were reported in the Netherlands; ribotype 027 was solely responsible for all, except one of the outbreaks in which ribotype 017 was the other predominant *C. difficile* strain. In the year 2005, several countries reported their first isolations of ribotype 027 strains: Poland (Pituch *et al.*, 2008), Japan (Kato *et al.*, 2007), Ireland (Long *et al.*, 2007) and Belgium (Joseph *et al.*, 2005). The first documented Irish case involved a patient transferred from the UK who was receiving antibiotic therapy. This index case was followed by related CDI clusters in two other hospitals in Ireland (Long *et al.*, 2007).

In 2006, France was struck by a *C. difficile* outbreak caused by ribotype 027 (Tachon *et al.*, 2006). The possibility that the strain had originated in Belgium was suggested as transfers between nursing homes and hospitals of the two countries are common and two of the patients had in fact been transferred from the same nursing home in Belgium. In the same year, *C. difficile* ribotype 027 was isolated in Tyrol, Austria from a British tourist (Indra *et al.*, 2006). The UK experienced yet another outbreak in 2006, this time at Maidstone and Tunbridge Wells (Healthcare Commision, 2007). Clusters of CDI caused by ribotype 027 were also seen in Belgium, to which ribotypes 078 and 031 also contributed (Kuijper *et al.*, 2008). Switzerland also witnessed an outbreak in which 17% of the isolates belonged to ribotype 027 (Fenner *et al.*, 2008a). Three hospitals in Denmark also suffered a ribotype 027 outbreak, in which, interestingly, another isolate that shared the genomic characteristics of ribotype 027, but was not ribotype 027, was also detected (Søes *et al.*, 2009). The same occurred in 10 hospitals in Luxembourg (Kuijper *et al.*, 2008). Spain reported its first ribotype 027 isolate in 2007; the index case was a patient transferred from the

UK and the second involved a laboratory worker (Kuijper *et al.*, 2008). By March 2007, Germany also reported its first ribotype 027 isolate (Zaiss *et al.*, 2007) which was followed by an outbreak that lasted till the September of that year (Kleinkauf *et al.*, 2007). First isolation of ribotype 027 was then reported in Finland (Lyytikäinen *et al.*, 2007), Hungary (Terhes *et al.*, 2009) and Norway (Ingebretsen *et al.*, 2008). The outbreaks across Europe continued with Northern Ireland (Aldeyab *et al.*, 2011), Finland (Kuijper *et al.*, 2008), two in Scotland (NHS, 2008; NHS, 2009), Austria (Indra *et al.*, 2009), Denmark (Bacci *et al.*, 2009), France (Birgand *et al.*, 2010) and ribotype 027 was also identified in Sweden (Kuijper *et al.*, 2008) and as far as Korea (Tae *et al.*, 2009), Hong Kong (Cheng *et al.*, 2009), Costa Rica (Quesada-Gómez *et al.*, 2010) and even Australia (Riley *et al.*, 2009); the Australian case is believed to be one of the first cases of travel-associated CDI. The extent of the global spread of *C. difficile* ribotype 027 today is remarkable (Fig. 6.1) (Clements *et al.*, 2010).

Fig. 6.1. Global distribution of *C. difficile* PCR ribotype 027



C. difficile PCR ribotype 027 (NAP1) has most severely affected North America and Europe. However, more recently it has been identified in South America, Asia and even Australia. Adapted from Clements *et al.*, 2010.

6.1.2. Increased morbidity and mortality?

Along with the increase in incidence of CDI during the emergence of ribotype 027, investigators the world over also noted increasing severity of disease and an increased case-fatality rate associated with this strain. Severe cases were defined as those having one or more of these symptoms within 30 days of diagnosis: pseudomembranous colitis, paralytic ileus, toxic megacolon, perforation or shock, colectomy, intensive care or death (Hubert *et al.*, 2007; Loo *et al.*, 2005; Morgan *et al.*, 2008; Pépin *et al.*, 2004).

One of the first studies to investigate disease severity was performed by Pépin and colleagues in Canada (Pépin *et al.*, 2004). A retrospective analysis of CDI over a period of thirteen years identified an almost 10-fold increase in annual incidence of CDI among people over 65 years of age. This corresponded to an increase in the number of severe cases from 6 to 10 per year between 1991 and 1998 with up to 71 cases per year in 2003. To better understand the effect of ribotype 027 strains on patient mortality, case-control studies were performed. During an outbreak in which ribotype 027 was the predominant strain, mortality among patients with CDI at 30 days was found to be 23% compared to just 7% in the control subjects (Pépin *et al.*, 2005b). In a similar study, 84% of the isolates were ribotype 027 and the crude mortality rate was approximately 25% (Loo *et al.*, 2005). A review of CDI across fourteen European countries found that more severe CDI was associated with the epidemic 027 ribotype and it was mostly healthcare associated (Barbut *et al.*, 2007).

The analysis of pre-epidemic and epidemic strains in Canada showed that the number of ribotype 027 strains isolated increased to 80% during the epidemic, whereas before the epidemic ribotype 001 was the predominant strain in about 20% of the hospitals investigated (Hubert *et al.*, 2007; Labbé *et al.*, 2008). A closer look at the genotype of the strains and disease severity by Hubert and co-workers found that severe disease was 2.3 times more likely in patients infected with ribotype 027 as compared to ribotype 001, while Labbé and colleagues found that this was true for mortality as well. There was however, no difference in the rate of recurrence between

the two strains (Labbé *et al.*, 2008). Similar observations were made in a hospital in England where it was observed that crude mortality and disease severity were higher in ribotype 027 infection - almost two to four times that of ribotype 106 infection - but recurrence rates were similar (Sundram *et al.*, 2009).

Although several case-control studies correlated ribotype 027 disease with increased morbidity and mortality, contradictory results were also observed. In a case-case study performed by Morgan and co-workers in which disease severity was associated with genotype, that is ribotype 027 versus non-027 ribotypes, 24% of patients with a ribotype 027 infection had severe CDI as compared to 17% of patients infected with a non-ribotype 027 strain, but this difference was not significant (Morgan *et al.*, 2008). In Germany, a comparison of isolates with disease severity did not show any greater effect of ribotype 027; ribotypes 001 and 078 were also associated with severe CDI and death (Arvand *et al.*, 2009). In Finland too, where ribotype 027 was most commonly associated with outbreaks and severe disease, it appeared to be linked with a higher 30 day case-fatality than other ribotypes but this was not significant after adjusting for age, suggesting that patient characteristics strongly influence disease severity (Kotila *et al.*, 2010). Similar observations were made by Verdoorn and co-workers who retrospectively studied strains with deletions in *tcdC* (Verdoorn *et al.*, 2009). They showed that morbidity and mortality were not greater in CDI caused by strains containing these deletions. However, the patients affected were older, had longer hospital stays and had prolonged antibiotic therapy. Another study gave a similar outcome where isolates with *tcdC* deletions, which were also binary toxin positive, were not significantly different from other strains with respect to disease severity, mortality or even length of hospitalisation (Goldenberg & French, 2011). The authors did find, however, that a greater immune response was mounted against these strains which was indicated by increased white blood cell counts and C-reactive protein. Thus, from all these studies it can be concluded that there is no definitive answer to whether ribotype 027 causes more severe disease than other strains, but it is evident that there are other risk factors involved that have led to the emergence of this ribotype as a global threat.

6.1.3. Risk factors

Over the years, several risk factors have been independently associated with *C. difficile* infection. These include patient characteristics such as age, especially above 65 years, immunosuppression, the presence of co-morbidities and a high leukocyte count (Pépin *et al.*, 2004). For ribotype 027 infections, disease severity was found to increase with age (Hubert *et al.*, 2007; Labbé *et al.*, 2008) but in patients less than 65 years of age and in those without any co-morbidities, the strain did not contribute to increased mortality, possibly due to the better immune status of young healthy individuals (Pépin *et al.*, 2005b).

In early studies, sex was not identified as a risk factor for CDI (Pépin *et al.*, 2005a; Pépin *et al.*, 2005b) but some found it to be a significant risk factor, with women being less prone to severe disease (Morgan *et al.*, 2008). This could be influenced by underlying conditions which could not be studied and were thus not included in the risk analysis. However, others also suggested that men were at higher risk of ribotype 027 infection (Weiss *et al.*, 2009), although there is no conclusive evidence to suggest that one sex is more prone to CDI than the other.

The duration of hospitalisation and previous incidence of CDI were found to be important risk factors (Pépin *et al.*, 2005a); patients spent a longer time in hospital, whether at first admission or on subsequent admissions (Pépin *et al.*, 2005b). In another study too it was found that, on average, case patients spent 10 days more in hospital than control patients (Weiss *et al.*, 2009). Further, the administration of proton pump inhibitors (PPIs), H_2 blockers and laxatives or chemotherapy were either found to be weakly associated with 027 CDI or not at all, while antacids and anti-motility drugs had no association (Loo *et al.*, 2005; Pépin *et al.*, 2005a; Sundram *et al.*, 2009).

Perhaps the most important risk factor associated with ribotype 027 is the use of antibiotics, the lack of which was found to be protective for CDI (Sundram *et al.*, 2009). The antibiotics associated with 027 CDI risk were second and third generation cephalosporins, macrolides, clindamycin, fluoroquinolones and intravenous β-

lactam/β-lactamase inhibitors (Pépin *et al.*, 2004; Pépin *et al.*, 2005a). In a case-control study, it was found that 20% more ribotype 027 cases had been exposed to antibiotics than controls (Loo *et al.*, 2005) and in a case-case study, it was found that 86% of patients had received at least one antibiotic in the two months before the diagnosis of CDI (Morgan *et al.*, 2008). In another study, although ribotype 027 only accounted for 6.2% of all the isolates in the study, it was found that approximately 78% of all the patients had had antibiotic treatment within the month before diagnosis of CDI (Barbut *et al.*, 2007). Total antibiotic usage was associated with an increase in the incidence of CDI caused by ribotype 027 (van der Kooi *et al.*, 2008).

Fluoroquinolones and cephalosporins were identified as the antibiotics related to the greatest risk of ribotype 027 infection (Loo *et al.*, 2005; Pépin *et al.*, 2005a; Weiss *et al.*, 2009). The exposure of patients to fluoroquinolones was significantly associated with ribotype 027 infection but not ribotype 001 infection; only 4% of the patients with ribotype 027 infection were previously exposed to clindamycin as compared to 29% of those with a ribotype 001 infection (Labbé *et al.*, 2008). A similar observation was made by Sundram and co-workers who found that the use of quinolones was associated more strongly with ribotype 027 rather than ribotype 106 infection (Sundram *et al.*, 2009). Exposure to moxifloxacin has also been linked to the subsequent development of CDI (Biller *et al.*, 2007).

Not just the antibiotic but the duration of use was also found to alter the risk of CDI. For quinolones, cephalosporins, clindamycin and macrolides, extended use increased the risk of CDI but, for cefoxitin, a single dose was more harmful (Pépin *et al.*, 2005a). Similarly, the use of ciprofloxacin for more than a week was linked to CDI but only to that caused by ribotype 027 (Sundram *et al.*, 2009). The strong correlation of fluoroquinolones with ribotype 027 explained the epidemic caused by ribotype 027 as it was found to be fluoroquinolone resistant but this association with fluoroquinolones was not seen in a later study in which second generation cephalosporins were found to be a risk factor (van der Kooi *et al.*, 2008). In a more recent study, neither cephalosporins, β-lactams nor fluoroquinolones appeared to be

significantly associated with CDI (Sundram *et al.*, 2009). Despite this uncertainty, the role of antibiotics in CDI is clear. The link between ribotype 027-induced CDI and antibiotics was further established when an approximately 50% decrease in the use of second and third generation cephalosporins, ciprofloxacin, clindamycin and macrolides resulted in about 60% less incidence of CDI (Valiquette *et al.*, 2007).

For the ribotype 027 epidemic, a combination of factors such as the increasing age of the population and the presence of co-morbidities, the change in antibiotic policies which included the extensive use of antibiotics, perhaps even the introduction of alcohol-based hand-washing resulted in the increase in incidence (Pépin *et al.*, 2005a; Pépin *et al.*, 2005b; Søes *et al.*, 2009). This, however, did not alter the fact that the responsible strain displayed increased virulence considering that patients with ribotype 027 disease required specific treatment with metronidazole or vancomycin (Barbut *et al.*, 2007).

6.1.4. What makes it hypervirulent?

6.1.4.1. Growth

The growth of the Canadian epidemic ribotype 027 strains showed similar growth kinetics to that of several toxinotype 0 isolates, although the cell density was always higher in ribotype 027 cultures over the 48 hours (Warny *et al.*, 2005). Despite the difference in cell density being significant at 24 hours, growth was rejected as a significant virulence factor. Additional studies also revealed that a NAP1a strain had a 20% higher optical density at 24 hours when compared to ribotype 001, VPI 10463 and other strains (Akerlund *et al.*, 2008).

6.1.4.2. Toxin production

Ribotype 027 was observed to produce greater amounts of toxin at a much faster rate (Warny *et al.*, 2005). On average, the ribotype 027 strains produced 16 times more toxin A and 23 times more toxin B than toxinotype 0 reference strains. The bulk toxin production observed in the logarithmic phase of growth in 027 isolates was a clear indicator of increased virulence; if these strains behaved similarly *in vivo*, it

would result in greater severity of disease. This increased severity could then be responsible for greater dissemination of spores in the environment. In another study measuring intracellular, extracellular and total toxin, the ribotype 027 isolates produced three to 13 times more toxin than other virulent strains (Akerlund *et al.*, 2008). Although more toxin was produced by ribotype 027 strains, the authors suggested that other factors could be involved in the hypervirulence of these strains.

6.1.4.3. Spore production

Spore production was also studied by Warny and co-workers (Warny *et al.*, 2005). They observed that at 48 and 72 hours, 93% of ribotype 027 cultures were positive for spores as compared to 72% of toxinotype 0 strains. The sporulation frequencies of ribotype 027 isolates at 24 hours were observed to be approximately 25% and up to 60% for some isolates (Akerlund *et al.*, 2008). Thus, a combination of greater toxin production and greater sporulation were suggested as factors for increased disease severity and greater transmission of ribotype 027, making it hypervirulent.

6.1.4.4. Genotype

The epidemic ribotype associated with the outbreaks in North America and Europe was characterised as PCR ribotype 027 strain, NAP1 (North American pulsed-field type 1), restriction endonuclease group BI and toxinotype III (MacCannell *et al.*, 2006; Warny *et al.*, 2005). Apart from being distinguished from other strains by established typing schemes (Clabots *et al.*, 1993; Gal *et al.*, 2005; O'Neill *et al.*, 1996; Rupnik *et al.*, 1998), ribotype 027 is also characterised by the presence of the binary toxin and by characteristic deletions found in *tcdC*, which have been suggested to confer hypervirulence upon this ribotype.

6.1.4.5. Presence of the binary toxin

The binary toxin of *C. difficile* was first identified in a ribotype 027 strain, CD196, isolated from a patient suffering from pseudomembranous colitis (Popoff *et al.*, 1988). The strain produced large amounts of this ADP-ribosylating toxin as well as toxin A and toxin B. Being a binary toxin, it has two genes *cdtA* and *cdtB*, which

code for the enzymatic CDTa and the binding CDTb components of the toxin, respectively (Perelle *et al.*, 1997). The binary toxin was found to be toxic to Vero cells and considered capable of inducing intestinal lesions. It was determined that although not necessary for virulence, the binary toxin could be an additional virulence factor for strains carrying the *cdtA* and *cdtB* genes such as ribotype 027 (Perelle *et al.*, 1997; Rupnik *et al.*, 2003a).

Strains of ribotype 027 characteristically carry the genes for the binary toxin, *cdtA* and *cdtB,* and their presence is a useful tool for differentiating these strains from those belonging to the more common toxinotype 0 strains (Warny *et al.*, 2005). It has been observed that the binary toxin genes are present only in strains belonging to variant toxinotypes, that is strains with significant polymorphisms in the toxin genes *tcdA* and *tcdB* when compared to toxinotype 0 strains like VPI 10463 (Stubbs *et al.*, 2000). Also, the amplification of *cdtB* is more efficient and thus, it alone can be used as a screening method. Screening of isolates from all the above-mentioned outbreaks and index cases described in 6.1.1 involved PCR amplification of at least *cdtB*.

6.1.4.6. Deletions in *tcdC*

On further characterisation, it was observed that in epidemic ribotype 027 strains, mutations are often present in the *tcdC* gene which is the negative regulator of toxin production in *C. difficile* (Smith, 2005). A study of the pathogenicity locus of a variety of *C. difficile* strains identified the presence of variant *C. difficile* strains with respect to *tcdC* (Spigaglia & Mastrantonio, 2002). In this study, three nucleotide sequences were described for the *tcdC* gene. These were named types A, B and C. *tcdC-A* shows a 39 bp deletion, while *tcdC-B* and *tcdC-C* show deletions of 18 bp. The epidemic strain in Quebec, Canada was found to carry an 18 bp deletion (Warny *et al.*, 2005).

Further analysis uncovered the presence of a single-base-pair deletion at position 117 (Δ117) (MacCannell *et al.*, 2006). The *tcdC* genotype containing the 18 bp deletion and Δ117 together (*tcdC-sc1*) was commonly identified in ribotype 027 strains isolated in several of the above-mentioned outbreaks (6.1.1); the 18 bp deletion was

used more frequently as a marker. Both the mutations were conserved across strains from different geographical areas (MacCannell *et al.*, 2006). During the epidemic in Canada, along with the 18 bp deletion, the 39 bp deletion was detected in some ribotype 027 isolates (Loo *et al.*, 2005). In Switzerland, the most commonly identified genotypes for ribotype 027 strains were *tcdC-A* and *tcdC-sc1* but a novel genotype with a 54 bp deletion (*tcdC-UHBS2*) was also found, although the implications of this deletion were unclear (Fenner *et al.*, 2008a).

The 39 bp deletion in *tcdC* leads to a truncated protein of 61 amino acids instead of the expected 232 amino acids and the 18 bp deletion results in a protein of 226 amino acids (Spigaglia & Mastrantonio, 2002). The deletion at position 117 induces a frameshift mutation in *tcdC*, truncating the TcdC protein to 65 amino acid residues (Curry *et al.*, 2007; MacCannell *et al.*, 2006). Thus, the 39 bp deletion and Δ117 have greater implications on the *tcdC* transcript and it was suggested that these deletions were responsible for the large amounts of toxin produced by ribotype 027.

6.1.4.7. Antibiotic resistance

The epidemic ribotype 027 isolates from North America and Europe were all found to be resistant to a range of fluoroquinolones including ciprofloxacin, moxifloxacin, gatifloxacin, ofloxacin and levofloxacin and erythromycin but susceptible to clindamycin, vancomycin and metronidazole (Barbut *et al.*, 2007; Hubert *et al.*, 2007; Joseph *et al.*, 2005; Kuijper *et al.*, 2006; Long *et al.*, 2007; Loo *et al.*, 2005; Pituch *et al.*, 2008; Zaiss *et al.*, 2007). This resistance to fluoroquinolones and an increased use of fluoroquinolones in hospitals for the treatment of respiratory tract infections are believed to have resulted in the emergence of the epidemic ribotype 027 strain (McDonald *et al.*, 2005; Pépin *et al.*, 2005a). However, ribotype 027 was not always resistant to fluoroquinolones. Genotype analysis and antibiotic susceptibility tests have revealed that the epidemic ribotype 027 (NAP1a) was resistant to fluoroquinolones, but historical 027 strains (NAP1b) were all susceptible, clearly indicating the acquisition of antibiotic resistance by this strain (Hubert *et al.*, 2007; McDonald *et al.*, 2005).

Before the emergence of the epidemic ribotype 027, the predominant ribotype isolated in epidemics was ribotype 001 which belongs to PFGE type J (Hubert *et al.*, 2007; Johnson *et al.*, 1999; Stubbs *et al.*, 1999). Ribotype 001 was found to be resistant to clindamycin which distinguished it from ribotype 027 (Climo *et al.*, 1998; Pear *et al.*, 1994). However, fluoroquinolone resistance has been observed in ribotype 001 too (Biller *et al.*, 2007). Clindamycin resistance was rarely reported in ribotype 027 (Kuijper *et al.*, 2006; Long *et al.*, 2007; McDonald *et al.*, 2005) until clindamycin-resistant 027 strains were identified in Ireland (Drudy *et al.*, 2008). Since then, intermediate resistance to clindamycin has been identified in the ribotype 027 isolate from the index case in Hungary (Terhes *et al.*, 2009), intermediate and complete resistance to clindamycin was observed among ribotype 027 isolates in Latin America (Quesada-Gómez *et al.*, 2010) and highly resistant strains have been isolated from an outbreak in Switzerland (Fenner *et al.*, 2008b). Interestingly, in Sweden, the moxifloxacin resistant isolates do not belong to ribotype 027, but to ribotype 012, which are also resistant to erythromycin and clindamycin (Norén *et al.*, 2010). Thus, different *C. difficile* ribotypes have overlapping resistance patterns and the hypervirulence of ribotype 027, although partially a result of resistance, is a multi-factorial phenomenon.

6.1.4.8. Genetic basis of antibiotic resistance

Fluoroquinolones are a group of antimicrobials that inhibit DNA synthesis through direct interaction with DNA complexed with DNA gyrase or with topoisomerase IV (Hooper, 2001). Resistance to fluoroquinolones occurs when there are mutations in the target enzymes, decreased entry of drugs into the bacterial cytoplasm or active release of the drug from the cytoplasm through efflux pumps. An early study of moxifloxacin resistant *C. difficile* strains identified mutations in *gyrA* at nucleotide 82 which leads to a threonine to isoleucine substitution in the GyrA protein but this mutation was not characteristic of all resistant strains, indicating that mutations in *parC* or efflux mechanisms were functional in *C. difficile* (Ackermann *et al.*, 2001). However, a later study also failed to detect the *parC* gene in the genome, suggesting that the genes for topoisomerase are absent in *C. difficile* (Dridi *et al.*, 2002). The

authors sequenced the quinolone resistance determining regions (QRDR) of *gyrA* (nucleotide 40 to 145) and *gyrB* (nucleotide 366 to 473). The mutations identified in GyrA were Thr82→lIe, Asp71→Val and Ala118→Thr, while those in GyrB were Asp426→Asn and Arg447→Leu. Most of these mutations have been previously identified in other bacteria and linked to fluoroquinolone resistance, except the *gyrA* mutation at nucleotide 71 (Hooper, 1999). Further, in a cluster of Irish ribotype 027 isolates, it was found that only the GyrA Thr82→lIe was associated with a high level of resistance, while *gyrB* remained mutation-free (Drudy *et al.*, 2007b). This mutation has been most commonly found in epidemic ribotype 027 isolates (Spigaglia *et al.*, 2008) but it has also been observed in other ribotypes (Carman *et al.*, 2009).

To study the induction of these mutations, Spigaglia and co-workers generated resistant mutants *in vitro* by selection using moxifloxacin and levofloxacin (Spigaglia *et al.*, 2009). Using moxifloxacin as the selective agent, high resistance was only observed after mutations in *gyrA* or *gyrB* were detected. Interestingly, in the epidemic 027 ribotype, the Asp81→Asn mutation in GyrA was only induced at the third step of the selection process. However, during selection with levofloxacin, at the second step, Leu451→Phe in GyrB was observed which was then followed by the more common Thr82→lIe in GyrA in the next step. These double mutations resulted in high fluoroquinolone resistance, although the significance of Leu451→Phe in GyrB in resistance is unknown. Similar observations correlating double mutations with high fluoroquinolone resistance, specifically Thr82→lIe along with another mutation, were made by Walkty and colleagues (Walkty *et al.*, 2010). Mutations in the DNA gyrase genes were not exclusive to ribotype 027 and reduced susceptibility to fluoroquinolones was also observed in the absence of any mutations, suggesting the presence of other mechanisms (Spigaglia *et al.*, 2009).

Resistance to clindamycin is associated with the presence of the *ermB* gene, a marker for transposon-mediated macrolide-lincosamide-streptogramin B resistance which was absent from epidemic ribotype 027 strains (Labbé *et al.*, 2008; MacCannell *et*

al., 2006). Interestingly, most ribotype 001 isolates were found to be clindamycin resistant and positive for *ermB* (Labbé *et al.*, 2008). However, recently, clindamycin resistant *ermB* positive ribotype 027 strains have been isolated (Drudy *et al.*, 2008), suggesting the acquisition of greater antibiotic resistance in this emerging ribotype.

6.1.5. Clonality

During the development of typing methods such as ribotyping and toxinotyping, it became evident that there was clonal diffusion of different strains of *C. difficile* in different parts of the world (Rupnik *et al.*, 1998; Stubbs *et al.*, 1999). To investigate this further and possibly identify hypervirulent clones, Lemée and colleagues used multilocus sequence typing (MLST) (Lemée *et al.*, 2004). However, they could not identify any such clones related specifically to either severe disease or particular geographical locations. They inferred that several stable *C. difficile* populations existed, exhibiting clonal population structure and suggested that the evolution of strains was guided by point mutations rather than recombinational exchange.

The clonal spread of ribotype 027 strains was, however, observed in several studies. Clusters of ribotype 027 isolates were observed in the Netherlands using multilocus variable-number tandem-repeat analysis (MLVA) (van den Berg *et al.*, 2007). It was observed that clusters with 100% similarity of isolates were hospital-specific but those from different outbreaks were only 86% similar. These groups however, were considerably dissimilar from a sporadic isolate. Further, the possibility of country-specific clusters was suggested; there was only 40% similarity between a UK isolate and several isolates from the Netherlands. MLVA was also used to identify clusters of CDI among 91 isolates from England (Fawley *et al.*, 2008). Twenty-three MLVA types were detected, of which 77% could be clustered to a single hospital and 95% to an institution. Two large clusters of CDI identified by surveillance information were confirmed by MLVA and two additional groups were identified. Specific sub-types could be linked to different incidences and trends in CDI could be identified. This clonal spread was also evident in Ireland (Solomon *et al.*, 2011). Using rep-PCR the investigators found that all ribotype 027 isolates from healthcare facilities grouped

into the same sub-type and this predominant sub-type was 89% similar to the NAP1 isolate from North America. Although heterogeneity existed among ribotype 027 isolates, they exhibited limited genetic diversity, suggesting adaptation to a particular niche and microevolution (Stabler *et al.*, 2006). However, infections by this clone have also been identified in animals (Songer *et al.*, 2009a).

The presence of a hypervirulent clade was confirmed by microarray analysis (Stabler *et al.*, 2006). The results showed that 20 of the 21 studied ribotype 027 strains formed a distinct lineage characterised by the presence of several deletions as compared to reference strain 630. They also identified clear differences between a historic ribotype 027 isolate, CD196, and an epidemic ribotype 027 isolate, R20291, and uncovered differences between these and strain 630 (Stabler *et al.*, 2009). Both ribotype 027 isolates contained the Δ117 and the 18 bp deletions in *tcdC* and were binary toxin positive, unlike 630 which could enhance virulence. They also had altered *tcdB* sequences in the 3' region which resulted in a considerably different TcdB protein but not a truncated one (Stabler *et al.*, 2008). However, this could affect toxicity and cell specificity. Also, the F1 flagellar locus in ribotype 027 strains is variable or lost, which could lead to greater motility (Stabler *et al.*, 2006). Moreover, the F2 flagellar region of ribotype 027 isolates contained glycosyl transferases that could affect autoagglutination. The ribotype 027 isolates were more motile than strain 630 and the epidemic ribotype 027 showed greater autoagglutination than the historic ribotype 027 isolate (Stabler *et al.*, 2009). The ribotype 027 isolates also contained an additional copy of the *arg* locus and additional regulatory genes that could be involved in *p*-cresol tolerance or production when compared to strain 630, which could aid survival.

Overall, ribotype 027 strains showed the presence of several deletions and inversions as compared to strain 630 which indicated significant adaptation that may have led to the emergence of the hypervirulent clone (Stabler *et al.*, 2010). Further, epidemic ribotype 027 appeared to have accumulated a variety of genetic elements such as putative antibiotic ABC transporter genes, putative cell surface proteins and a toxin-

antitoxin system which could explain the increased virulence linked to it in humans and in animal models (Razaq *et al.*, 2007).

The primary aim of this study was to characterise seven ribotype 027 isolates from Scotland and five outbreak ribotype 027 isolates from the Netherlands and determine if these clusters of isolates shared any similarities. The secondary aim was to extend the phenotypic and genetic characterisation studies to include ribotypes 001 and 106, which are currently predominant in Scotland, and strain 630, belonging to the previously dominant ribotype 012, in order to identify traits that might contribute to ribotype 027 being more virulent than these successful strains.

6.2. Methods

The strains used in this study comprised seven isolates from Scotland (4868-83) and five isolates from the Netherlands (4828-4832) collected from outbreaks and recognised as ribotype 027 (Table 2.1). *C. difficile* strains 630 and VPI 10463 were used as reference strains and the representative isolates of ribotypes 027, 001 and 106 (2.1.2 and chapter 3) were also included. The phenotypic characteristics of the isolates such as growth, toxin production, spore production, motility and autoagglutination (2.2.1-2.2.7) were studied first, followed by S-layer typing (2.2.8), ribotyping, toxinotyping, binary toxin detection and flagellum analysis (2.3.2-2.3.5). Further, the *tcdC* gene of the isolates was amplified by PCR (2.3.6) along with the *gyrA* and *gyrB* genes of the binary toxin (2.3.10). These amplified products were sequenced (2.3.13) to identify any chromosomal mutations carried by these strains. Finally, antibiotic susceptibility testing was performed on all the isolates (2.8.2) and the results were interpreted using the values listed in Table 2.4.

6.3. Results

6.3.1. Growth

Growth of all the *C. difficile* isolates was determined by measuring the OD_{600} at 48 h. There was no marked difference between the growth of the reference strains and the ribotype 027 strains or between the Scottish and Dutch isolates (Fig. 6.2.a). Thus, a

greater growth rate or more cell numbers were not identified as contributory factors to the virulence of ribotype 027 strains.

6.3.2. Toxin production

Total toxin (A+B) production was measured by ELISA and it was found that the ribotype 027 strains produced considerably greater amount of total toxin at 48 h as compared to strain 630 and even produced more toxin than VPI 10463 (Fig. 6.2.b). There was no difference in toxin production between the two ribotype 027 groups. The isolates belonging to the epidemic ribotypes 001 and 106 produced markedly less toxin than ribotype 027 isolates.

6.3.3. Sporulation

Spore production varied slightly between the ribotype 027 isolates. However, they all produced greater number of alcohol-resistant spores than strain 630 and even ribotype 001 (Fig. 6.2.c). The average spore production of all the ribotype 027 isolates was similar to ribotype 106. VPI 10463 was the lowest spore producer.

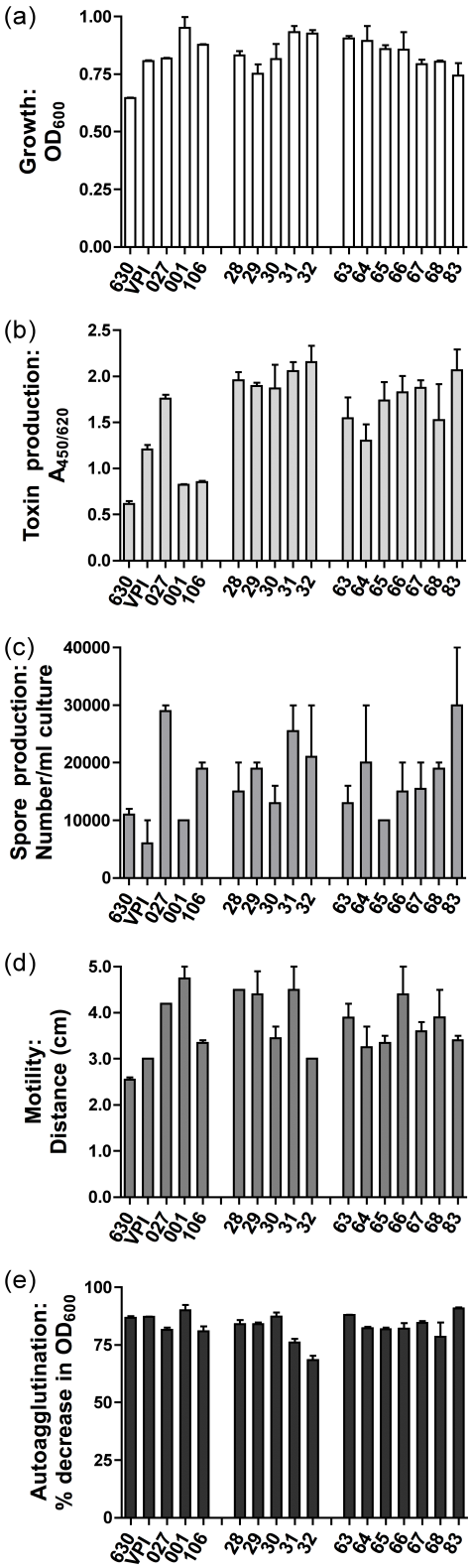
6.3.4. Motility assay

All the *C. difficile* strains tested were found to be similarly motile (Fig. 6.2.d). From the length of growth observed in 0.05% BHI agar, it was determined that on average, ribotype 027 strains were more motile than reference strains 630 and VPI 10463. The ribotype 106 isolate was found to be as motile as ribotype 027, however, the ribotype 001 isolate demonstrated the greatest motility.

6.3.5. Autoagglutination assay

Although slight variations in the percentage of autoagglutination were observed between some ribotype 027 isolates, there was no marked difference between the strains of the ribotypes studied (Fig. 6.2.e).

Fig. 6.2. Phenotypic characteristics of ribotype 027 isolates, the reference strains and isolates belonging to ribotypes 001 and 106

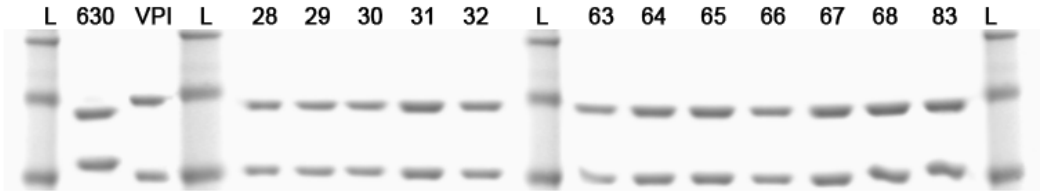


(a) No marked difference in growth was observed among the isolates of ribotype 027 at 48 h. (b) The isolates did, however, produce greater amounts of total toxin (A +B) as compared to the reference strain 630 and the isolates of ribotypes 106 and 001. (c) Spore production was found to be variable among ribotype 027 isolates, but on average was much higher than the reference strains. The isolate belonging to ribotype 106 showed the same level of spore production as the average of the ribotype 027 isolates combined. (d) All the strains in this study were motile. The ribotype 001 isolate was found to be slightly more if not as motile as the ribotype 027 strains. (e) There was no marked difference in the autoagglutination observed between the different isolate types. From these results, it would appear that although increased growth does not contribute to the hypervirulence of ribotype 027, high toxin and spore production and perhaps increased motility are the factors that cause the altered behaviour of this ribotype.

6.3.6. S-layer typing

S-layer typing of the strains showed that all ribotype 027 isolates belonged to the same S-layer type (Fig. 6.3). All the strains had a high molecular weight SLP of 54 kDa and a low molecular weight SLP of 38 kDa, classifying them as S-layer type 5438.

Fig. 6.3. S-layer typing of isolates in this study

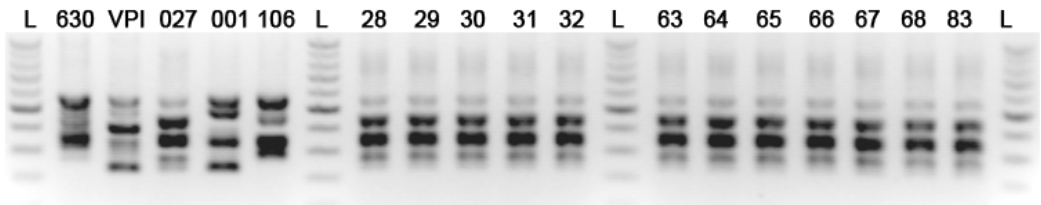


The S-layer proteins extracted with GHCl were run on an SDS-PAGE gel to obtain the above banding pattern. All ribotype 027 isolates had the same pattern with a low molecular weight SLP of 38 kDa and a high molecular weight SLP of 54 kDa. Thus, the assigned S-layer time for ribotype 027 was 5438.

6.3.7. Ribotyping

Ribotyping was performed and, as expected, the banding patterns of the reference strains and the isolates belonging to ribotypes 027, 001 and 106 were all different from each other (Fig. 6.4). The patterns obtained for the isolates from Scotland and the Netherlands were identical and matched precisely to that of ribotype 027 in the laboratory database, confirming that all these isolates belonged to PCR ribotype 027.

Fig. 6.4. Ribotyping of isolates in this study

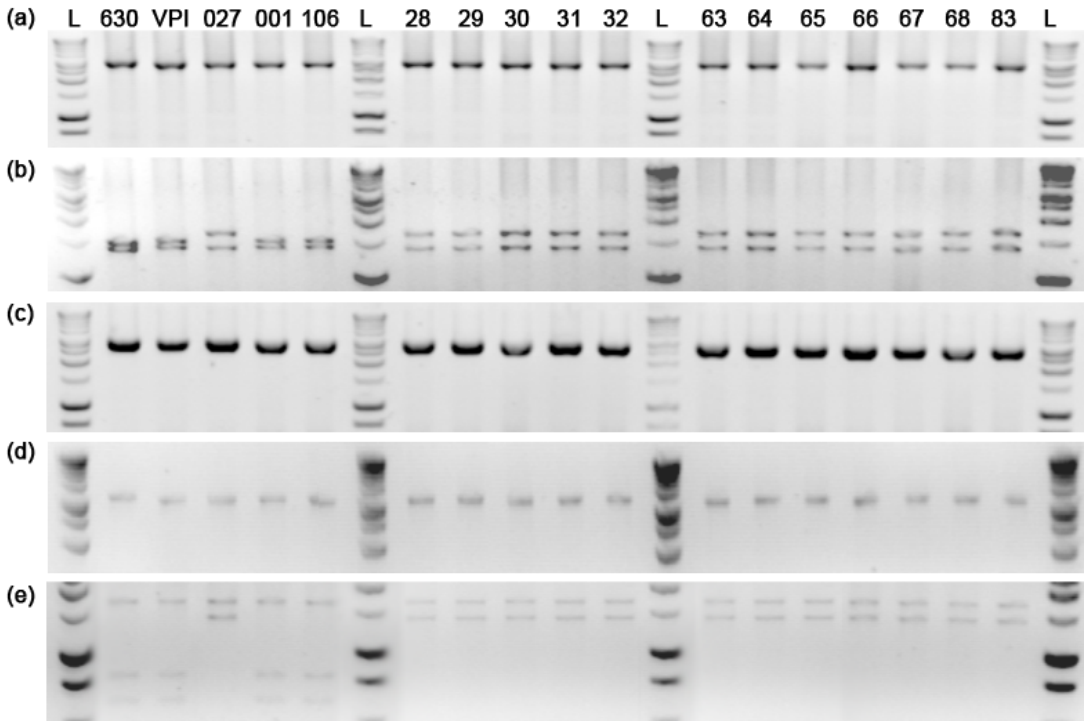


The amplification of the 16S-23S rRNA intergenic spacer region revealed identical bands for all ribotype 027 isolates, which matched reference isolates of the same ribotype.

6.3.8. Toxinotyping

The 3.1 kb products expected for the A3 and B1 regions of the toxin genes *tcdA* and *tcdB* respectively were found in all the strains tested (Fig. 6.5). From the restriction of these products and the patterns obtained, it was confirmed that all ribotype 027 isolates belonged to toxinotype III.

Fig. 6.5. Toxinotyping of isolates in this study

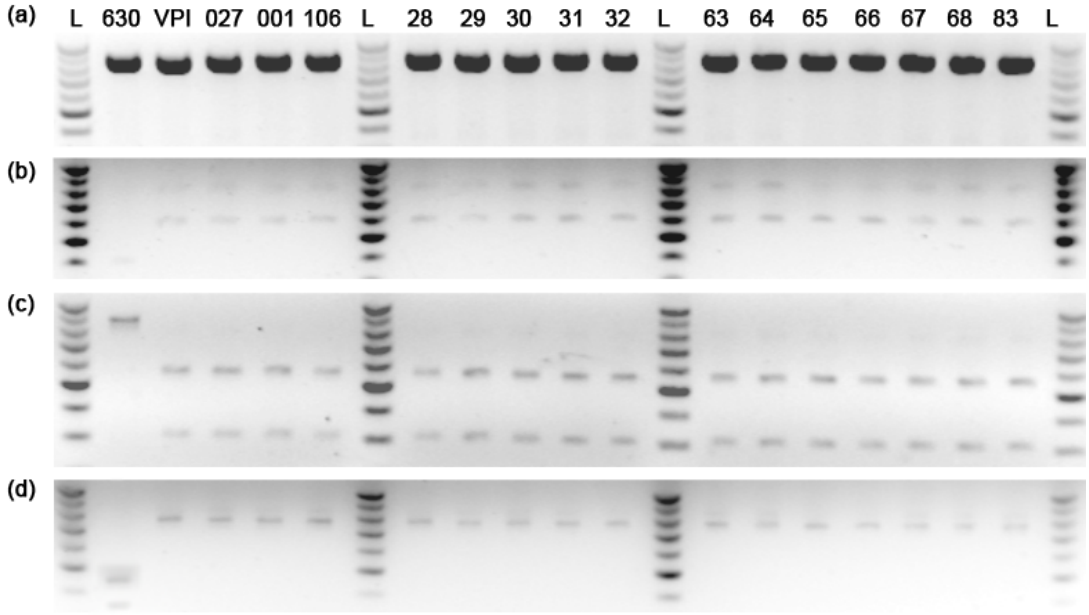


(a) *tcdA* and (c) *tcdB* were amplified by PCR to give products of 3.1 kb. Restriction of (b) *tcdA* with *Eco*RI gave pattern 2 and *tcdB* with (d) *Acc*I and (e) *Hin*cII gave pattern 4. The resultant type for all ribotype 027 isolates was thus determined as toxinotype III.

6.3.9. Flagellum analysis

PCR amplification of *fliC* in all the strains gave a product of 870 bp (Fig. 6.6). For all ribotype 027 strains, the restriction pattern obtained was bbb, putting them in RFLP group VII. Group VII strains belong to serogroup A/B, both of which correspond to toxinotype III.

Fig. 6.6. Analysis of the *fliC* gene and flagellar typing of isolates in this study

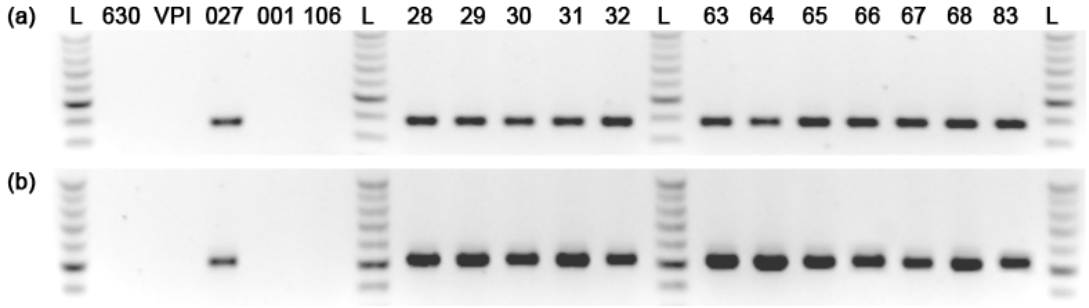


(a) The *fliC* gene was amplified by PCR to give a product of 870 bp. The product was then restricted with (b) *Rsa*I, (c) *Hin*dIII and (d) *Hpa*I. All ribotype 027 strains belonged to *fliC* group VII which corresponds to toxinotype III and ribotype 027.

6.3.10. Binary toxin detection

All ribotype 027 isolates were found to be binary toxin positive; 375 bp bands were observed for *cdtA* and 510 bp bands for *cdtB* (Fig. 6.7). The reference strains and the isolates of ribotypes 106 and 001 did not show the presence of either of the binary toxin genes as expected.

Fig. 6.7. Detection of the binary toxin genes of isolates in this study



All ribotype 027 isolates were positive for the binary toxin genes (a) *cdtA* and (b) *cdtB*.

6.3.11. PCR amplification and sequencing of *tcdC*

The expected 718 bp *tcdC* band was observed in all the isolates (Fig. 6.8.a). The sequencing of *tcdC* revealed that all ribotype 027 isolates carried the Δ117 mutation (Fig. 6.8.b) which was not present in the reference strains or in ribotypes 001 and 106 (Fig. 6.8.c). Ribotype 027 isolates also carried the characteristic 18 bp deletion from nucleotides 330 to 347 (Fig. 6.8.d) which was also not observed in the other strains (Fig. 6.8.e). Hence, the truncated TcdC protein was found only in the isolates belonging to PCR ribotype 027 (Fig. 6.8.f and 6.8.g).

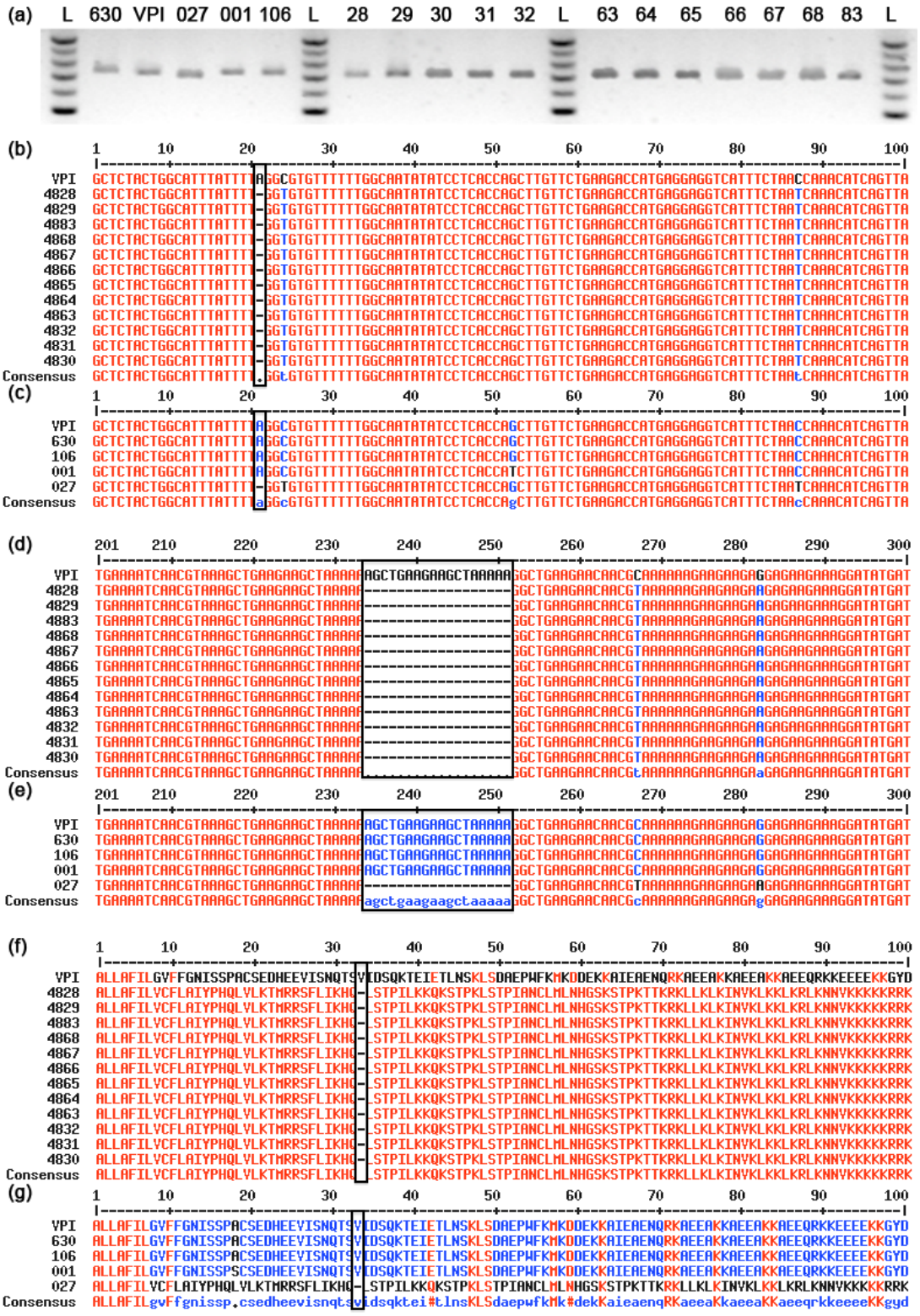
6.3.12. PCR amplification and sequencing of *gyrA* and *gyrB*

The *gyrA* and *gyrB* genes were amplified from all the isolates, giving bands of 633 bp (Fig. 6.9.a) and 514 bp (Fig. 6.9.f), respectively. All the isolates belonging to ribotype 027 had a mutation in the *gyrA* gene (Fig. 6.9.b). This mutation was also found in the isolates of ribotypes 106 and 001 (Fig. 6.9.c). This mutation leads to a Thr82→Ile substitution in the GyrA protein (Fig. 6.9.d,e) which is commonly linked to increased fluoroquinolone resistance. No mutations were observed in the *gyrB* gene of any ribotype.

6.3.13. Antimicrobial susceptibility testing

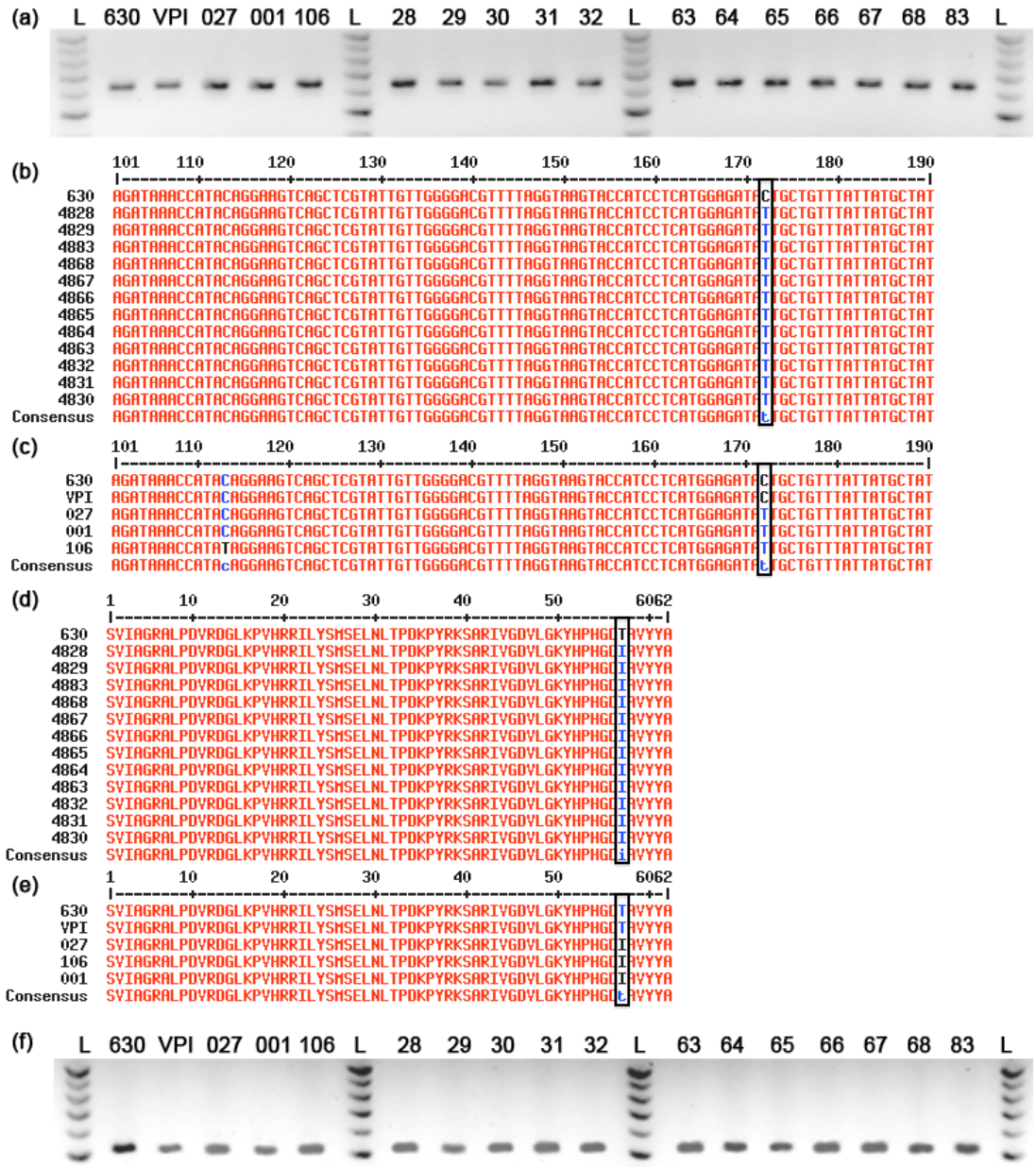
By performing antimicrobial susceptibility tests, minimum inhibitory concentrations (MICs) for the different isolates were obtained for each antibiotic. These values were then interpreted using CLSI criteria and the isolates were classified as being susceptible, resistant or of intermediate resistance to a particular antibiotic as appropriate (Table 6.1). All the isolates tested were found to be susceptible to vancomycin (V), metronidazole (M) and tetracycline (T). All the ribotype 027 isolates showed intermediate resistance to ceftriaxone (C) and were resistant to clindamycin (CL), erythromycin (E), moxifloxacin (MX) and ciprofloxacin (CP). The isolates of ribotypes 001 and 106 were resistant to ceftriaxone, but showed the same sensitivities to the other antibiotics as ribotype 027, while the reference strains differed from the ribotype 027 isolates by being sensitive to moxifloxacin and ciprofloxacin.

Fig. 6.8. Amplification and sequencing of *tcdC* of isolates in this study



(a) *tcdC* was successfully amplified by PCR in all strains. Only ribotype 027 isolates carried (b, c) a Δ117 and (d, e) an 18 bp deletion in *tcdC*, leading to (f,g) a truncated TcdC protein.

Fig. 6.9. Amplification and sequencing of *gyrA* and *gyrB* of isolates in this study



The (a) *gyrA* gene in all the isolates was amplified by PCR, followed by sequencing. (c) Analysis of the obtained gene sequences identified a mutation in *gyrA* in all isolates of ribotype 027 and (d) also in the isolates of ribotype 106 and ribotype 001, but not in strains 630 or VPI 10463. (d, e) This ACT→ATT mutation caused the Thr82→Ile substitution in the GyrA protein in these ribotypes, which is associated with fluoroquinolone resistance. (f) The *gyrB* gene was also amplified in all isolates but no mutations were observed in the gene sequence of any ribotype (not shown).

Table 6.1. Antimicrobial susceptibility testing of isolates in this study

	Antibiotics tested[b] and the inferred susceptibility of the isolates to them[c]							
Strain	V	M	C	CL	T	E	MX	CP
Strain 630	S	S	I	R	S	R	S	S
Strain VPI 10463	S	S	I	R	S	R	S	S
Ribotype 027	S	S	I	R	S	R	R	R
Ribotype 001	S	S	R	R	S	R	R	R
Ribotype 106	S	S	R	R	S	R	R	R
4828	S	S	I	R	S	R	R	R
4829	S	S	I	R	S	R	R	R
4830	S	S	I	R	S	R	R	R
4831	S	S	I	R	S	R	R	R
4832	S	S	I	R	S	R	R	R
4863	S	S	I	R	S	R	R	R
4864	S	S	I	R	S	R	R	R
4865	S	S	I	R	S	R	R	R
4866	S	S	I	R	S	R	R	R
4867	S	S	I	R	S	R	R	R
4868	S	S	I	R	S	R	R	R
4883	S	S	I	R	S	R	R	R

[b] The antibiotics used in this study were: V= Vancomycin, M= Metronidazole, C= Ceftriaxone, CL= Clindamycin, T= Tetracycline, E= Erythromycin, MX= Moxifloxacin, CP= Ciprofloxacin

[c] Using the guidelines and from the MIC values obtained, the isolates were classified as: S= Susceptible, I= Intermediate, R= Resistant

Ribotype 027 isolates were all resistant to erythromycin, clindamycin, moxifloxacin and ciprofloxacin but were sensitive to vancomycin and metronidazole. They all showed intermediate resistance to ceftriaxone.

6.4. Discussion

Ever since the emergence of the hypervirulent epidemic *C. difficile* ribotype 027, characterisation studies have been carried out in several countries that have experienced outbreaks caused by this strain. Ribotype 027 epidemics were identified in the UK from 2004 onwards but these were limited to England and Wales (Brazier *et al.*, 2007). The first Scottish ribotype 027 isolate was identified in 2006 (Kuijper *et al.*, 2007) when ribotype 106 and 001 were the predominant strains; the frequency of isolation of ribotype 027 in Scotland increased from the beginning of 2008 (Wiuff *et al.*, 2011). By the end of 2008, our laboratory collection included seven 027 isolates from four hospitals across Scotland. By characterisation of these strains and by comparison and contrast with other predominant ribotypes and outbreak isolates, an insight into the virulence of this ribotype was obtained.

As previously described for other ribotype 027 isolates, both the Scottish and Dutch isolates showed similar characteristics. As there was no historic isolate in this study, comparisons were made with strains 630 and VPI 10463 and ribotypes 001 and 106. Ribotype 027 isolates did not show increased growth at 48 hours, but produced markedly more toxin than the other strains, as shown by Warny and colleagues for the Canadian epidemic strain (Warny *et al.*, 2005). Also, they showed increased sporulation capacity compared with the other strains, except ribotype 106. This is also a trait of the epidemic ribotype 027 strain that had been previously reported (Akerlund *et al.*, 2008). They also exhibited greater motility than the reference strains, but not the other predominant ribotypes 001 and 106; in fact, ribotype 001 was found to be the most motile strain. Increased motility in ribotype 027 strains compared to strain 630 has also been observed with both a historic ribotype 027 isolate and one epidemic strain from both the UK and North America (Stabler *et al.*, 2009). Although significant differences in autoagglutination were also found between ribotype 027 strains and strain 630 (Stabler *et al.*, 2009), none were identified in this study. Of all the phenotypic traits studied here, it appears as though increased toxin production is the only one that contributes to the difference in virulence observed between ribotype 027 and the other ribotypes.

All the Scottish and Dutch isolates were characterised as ribotype 027, toxinotype III, S-layer type 5438 and *fliC* group VII. They were all binary toxin positive, unlike strains 630 and VPI 10463 and ribotypes 001 and 106 (Rupnik *et al.*, 2003a; Stubbs *et al.*, 2000). The binary toxin is considered to be an additional virulence factor in *C. difficile* and its absence from the other ribotypes identified it as a factor in the greater virulence of ribotype 027. All ribotype 027 isolates also had deletions in *tcdC*, the negative regulator of toxin production (Matamouros *et al.*, 2007) and showed the *tcdC-sc1* genotype which has both the 18 bp deletion from nucleotide positions 330 to 347 and the frameshift mutation at position 117 (MacCannell *et al.*, 2006). These deletions, which result in a truncated TcdC protein of 65 amino acid residues have been linked to the high toxin levels produced by ribotype 027 strains. The non-ribotype 027 strains were all binary toxin negative and did not have any mutations in the *tcdC* gene, which clearly differentiated them from ribotype 027 isolates.

Ribotype 027 isolates were all resistant to clindamycin, erythromycin, moxifloxacin and ciprofloxacin, but sensitive to vancomycin and metronidazole. The same antibiotic sensitivity pattern was observed for ribotypes 001 and 106, except that they were also resistant to ceftriaxone, while ribotype 027 isolates showed intermediate resistance to this antibiotic. Not surprisingly, the ACT→ATT mutation in *gyrA* resulting in the Thr82→lIe substitution in the GyrA protein, which is linked to fluoroquinolone resistance was observed in all ribotype 027 isolates (Ackermann *et al.*, 2001). However, this mutation was not exclusive to ribotype 027; it was also observed in ribotypes 001 and 106. No mutations in the *gyrB* gene were detected in any of the strains, despite the high level of resistance observed. It has previously been observed that ribotypes 027, 001 and 106 had high levels of resistance to erythromycin and moxifloxacin and higher MIC values for metronidazole (Brazier *et al.*, 2008). Further, fluoroquinolone resistance has also been seen in ribotypes 001 and 106 (Balassiano *et al.*, 2009; Walkty *et al.*, 2010) and the GyrA mutation Thr82→Ile has been detected in ribotype 001 (Saxton *et al.*, 2009).

The change in epidemiology associated with the emergence of ribotype 027 was noted for the severity of disease and the ability to affect healthy individuals (Centers for Disease Control and Prevention, 2005). It also correlated with an increased use of antibiotics, especially fluoroquinolones (Pépin *et al.*, 2005a). The epidemic ribotype 027 strain exhibited high toxin and spore production (Akerlund *et al.*, 2008; Warny *et al.*, 2005). It was suggested that these characteristics and the acquisition of resistance to antibiotics by ribotype 027 led to the superimposition of epidemic ribotype 027 over the previously predominant ribotype 001 (Labbé *et al.*, 2008). The epidemiology of CDI, however, is still changing; highly virulent strains other than ribotype 027 also exist (Cartman *et al.*, 2010). Ribotype 001 has been responsible for several large outbreaks (Graf *et al.*, 2009; Johnson *et al.*, 1999). Ribotype 106 is now a dominant strain in the UK, replacing ribotype 001 (Brazier *et al.*, 2007; Brazier *et al.*, 2008). Ribotype 078 has been identified as a high toxin producer *in vitro* (Jhung *et al.*, 2008); ribotype 017 has been associated with lethal CDI (Arvand *et al.*, 2009) and increased sporulation has been identified in ribotype 002 (Cheng *et al.*, 2011).

Lemée and colleagues postulated that for the existence of hypervirulent lineages in *C. difficile*, the horizontal exchange of genes between strains was necessary (Lemée *et al.*, 2004). Since the hypervirulent ribotype 027 lineage does exist (Stabler *et al.*, 2006) we can presume that genetic exchange between *C. difficile* strains is ongoing, explaining why previously identified ribotype 027-specific markers can be identified appear to be distributed amongst other ribotypes to varying degrees (Marsden *et al.*, 2010); ribotype 078 is binary toxin-positive and has deletions in the *tcdC* gene. From the results presented here, it can be concluded that there has been a clonal spread of the hypervirulent ribotype 027 across Europe. Ribotype 027 isolates from Scotland and the Netherlands share these properties with the epidemic strain first seen in Canada. However, this study did not include in-depth genomic analysis of the strains which could estimate the degree of similarity between the strains or identify genomic regions that could enhance virulence. Although the epidemic *C. difficile* ribotype 027 is hypervirulent compared to previously common strains, all *C. difficile* strains should be considered to be equally dangerous pathogens.

7. Conclusions

Clostridium difficile is a fascinating pathogen. Although it was identified as the causative agent of CDI and pseudomembranous colitis in the late 1970s, the mechanisms involved in its virulence are still being discovered today. One of the most significant advances in *C. difficile* studies has been the ClosTron system which enables us to knock-out genes from *C. difficile* to isolate the effects of individual virulence factors (Heap *et al.*, 2007). However, in the absence of genetic manipulation systems, a comparison of strains of different pathogenic potential is useful to gain insight into the variations that affect the outcome of infection. In the studies included in this thesis, this approach has been used to identify differences between historic and epidemic strains of *C. difficile* in several different settings.

The five main *C. difficile* strains studied were selected based on the epidemiology of CDI in Scotland. Ribotype 012, which includes strain 630, was once prevalent in the region and represented an historic isolate. Ribotypes 106 and 001 were the most common locally endemic strains when these studies began. Ribotype 027 was the epidemic hypervirulent strain that steadily increased in prevalence over the duration of these investigations. Lastly, VPI 10463 was used as a reference strain to facilitate comparisons with previous studies.

The expression of toxins and spores was found to be significantly different between these five strains, although their patterns of growth were similar. The production of toxin A and toxin B were highest in ribotype 027, followed by ribotypes 106, 001 and 630, respectively. VPI 10463 produced almost equivalent amounts of toxin to ribotype 027 confirming previous observations. The production of spores was highest in ribotype 106, followed by ribotype 027, ribotype 001 and strain 630, respectively. VPI 10463 produced the least number of spores, also agreeing with previous studies. Thus, ribotypes 027 and 106 both demonstrated a more virulent phenotype than the other strains, which correlates with the increased incidence and severity of disease recently associated with them. This could also be a reason why ribotype 001 was

replaced by ribotype 106 as the most prevalent strain in Scotland. Thus, it is evident that increased toxin and spore production were advantageous in virulence.

These inter-strain differences in phenotype were also observed at the genetic level. Not surprisingly, transcription studies showed that expression of the toxin genes, *tcdA* and *tcdB*, was higher in the strains that produced most toxin. The level of *tcdR* expression was also slightly higher in ribotypes 027 and 106 at 24 hours, suggesting that this auto-regulated gene was positively inducing toxin production even in the late stationary phase of growth. A more significant observation was that the expression of *tcdC*, which negatively regulates toxin production, did not decrease over time as expected and previously observed and also expected from a negative regulator. This observation was not limited to the higher toxin producers and thus suggested that *tcdC* had a modulatory rather than a repressive effect on toxin production. The expression of *tcdE* however, was highest in ribotype 027 and VPI 10436 and could be responsible for the high levels of toxin detected extracellularly in cultures of these strains. Increased toxin production coupled with increased toxin release could contribute to the hypervirulence of ribotype 027. Another important observation was that *spo0A* expression in ribotypes 106 and 027, the highest spore producers, remained high over the early exponential phase. This indicated a longer duration of activation of the sporulation cycle, which could result in greater numbers of spores being produced. This was another contributory factor in the virulence of the epidemic strains. Although these phenotypic and genetic differences were identified *in vitro*, they could mirror the virulence of the strains *in vivo*.

The susceptibility to disinfectants also varied between strains. Vegetative cells and spores of all five strains were effectively neutralised under laboratory conditions, but not under stimulated 'real-life' conditions. Although neutralisation was observed, the epidemic ribotypes were more resistant to higher concentrations of the agents as compared to strain 630. For some agents, just a 2-fold dilution lower than the recommended concentration could be ineffective, emphasising the need to reconstitute disinfectants properly before use. Moreover, whether this neutralisation

was the result of cell-death or merely dormancy was not identified. The focus of these studies, however, was on the more practical aspect of disinfection - that of surfaces. Of the agents studied, only chlorine-based Actichlor was suitable for the decontamination of hard, non-porous surfaces but between two and ten minutes' exposure was required for the complete elimination of *C. difficile* spores. The other agents, all of which are used in laboratories, showed poor efficacy which decreased even further in the presence of organic matter. Here too, spores of the epidemic ribotypes 027, 001 and 106 were more resistant to the agents. The use of chlorine-based agents is not preferred due to their side-effects, but on the other hand, surface application of disinfectants for prolonged periods of time is not practical. Actichlor is commonly used in hospitals for intensive cleaning but there is a need for a similar disinfectant in the laboratory. Laboratory-acquired CDI is not unheard of (Hell *et al.*, 2009) and, from the spread of *C. difficile* detected in and around the laboratory in this study, it is evident that workers can be exposed to the bacterium even away from the work-bench. If spores of epidemic strains are more resistant to disinfection, it implies that they persist longer in the environment which would increase the incidence of disease caused by them.

The response of the strains to sub-inhibitory concentrations was also found to be variable. Exposure to such dilutions of non-chlorine agents was investigated and it was observed that such exposure did not affect the growth of the strains but dramatically increased sporulation in the epidemic ribotypes. This effect was most marked in ribotype 001 in agreement with previous observations. These observations suggest that sub-inhibitory concentrations of disinfectants could lead to the increased dissemination of spores in the environment, which would also affect the incidence of disease by such ribotypes. Although the significance of exposure to sub-inhibitory concentrations of disinfectants *ex vivo* on virulence *in vivo* is unknown, it suggests that under stressful - but not cidal - conditions they could affect the phenotype of *C. difficil*e strains. From these results, it was evident that epidemic ribotypes were more likely to persist and spread in the environment.

The interactions of *C. difficile* proteins with macrophages were not variable between strains. The toxins, surface-layer proteins, flagella and heat-shock proteins of the five strains were all able to stimulate production of pro-inflammatory cytokines. Despite marked differences in the amounts of toxin produced between the strains during growth, the immune response to culture supernatants was similar. Also, there were no strain-specific differences in the cytokine response to other proteins. This suggested that surface-associated proteins of *C. difficile* have the potential to elicit inflammatory responses irrespective of toxin production and may contribute to the inflammation and ulceration observed in CDI.

The adherence of the strains to epithelial cells did vary. The epidemic strains were more adherent than strain 630 to all types of epithelial cells used in the study, but surprisingly, VPI 10463 showed the most attachment. The flagella and S-layer proteins of the strains were found to contribute equally to the levels of attachment of the strain from which they were extracted. In some cases, these proteins were found to notably increase adherence of *C. difficile* cells, possibly by the formation of extracellular webs or by inducing autoagglutination. These preliminary studies showed that surface-proteins of *C. difficile* may influence the physical binding of bacteria to the epithelium and also the immune responses that follow.

A study of ribotype 027 strains showed that certain characteristics were conserved within this group. They exhibited the same phenotype, were identical by molecular and phenotypic typing methods and showed identical deletions in *tcdC* and *gyrA*. The strains were binary toxin positive and more resistant to antibiotics than ribotypes 001 and 106 and strains 630 and VPI 10463. The deletions in *tcdC*, especially the frameshift mutation at position 117, could contribute to the hypervirulence of ribotype 027. Mutations in *gyrA* associated with increased antibiotic resistance were observed in all the hypervirulent isolates but were not exclusive to ribotype 027; ribotypes 001 and 106 carried identical mutations. Thus, the epidemic strains had genetic potential for greater antibiotic resistance.

In conclusion, the sum of observations from the studies in this thesis suggests that there have been genetic and phenotypic changes in *C. difficile* strains over time. Together, factors such as enhanced expression of virulence factors, longer persistence in the environment, increased adherence *in vivo* and greater antibiotic-resistance may have resulted in increased virulence and a subsequent increase in the prevalence of the currently epidemic *C. difficile* strains. However, only single isolates from each ribotype were used in the three main studies. It is, thus, possible that variations exist within ribotypes. Similar experiments with multiple isolates of the same ribotype could lead to a better understanding of the virulence of *C. difficile*.

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Comparison of toxin and spore production in clinically relevant strains of *Clostridium difficile*

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Clostridium difficile is a major cause of nosocomial diarrhoea. The toxins that it produces (TcdA and TcdB) are responsible for the characteristic pathology of *C. difficile* infection (CDI), while its spores persist in the environment, causing its widespread transmission. Many different strains of *C. difficile* exist worldwide and the epidemiology of the strains is ever-changing: in Scotland, PCR ribotype 012 was once prevalent, but currently ribotypes 106, 001 and 027 are endemic. This study aimed to identify the differences among these ribotypes with respect to their growth, and toxin and spore production *in vitro*. It was observed that the hypervirulent ribotype 027 produces significantly more toxin than the other ribotypes in the exponential and stationary phases of growth. Further, the endemic strains produce significantly more toxins and spores than ribotype 012. Of note was the observation that *tcdC* expression did not decrease into the stationary phase of growth, implying that it may have a modulatory rather than repressive effect on toxin production. Further, the increased expression of *tcdE* in ribotype 027 suggests its importance in the release of the toxins. It can thus be concluded that several genotypic and phenotypic traits might synergistically contribute to the hypervirulence of ribotype 027. These observations might suggest a changing trend towards increased virulence in the strains currently responsible for CDI.

INTRODUCTION

Clostridium difficile is a Gram-positive, anaerobic, spore-forming bacillus that was first identified as the cause of antibiotic-associated pseudomembranous colitis in 1978 (Bartlett *et al.*, 1978; George *et al.*, 1978; Larson *et al.*, 1978). Today, it is the most common cause of nosocomial diarrhoea. Once mainly associated with the use of antibiotics and being elderly, it is now also found in young, previously healthy adults with no history of antibiotic usage (McFarland *et al.*, 2007). *C. difficile* is acquired from the environment in the form of spores and transmitted by the faecal–oral route. The organism is able to colonize the gut when the normal protective flora is disrupted by the use of broad-spectrum antibiotics. Once the infection is established, the bacterium produces two large Rho glucosylating exotoxins, toxin A (TcdA), an enterotoxin, and toxin B (TcdB), a cytotoxin, which result in the characteristic pathology of *Clostridium difficile* infection (CDI). Though asymptomatic carriage of *C. difficile* is common (Riggs *et al.*, 2007), the presentation of CDI can vary from mild self-limiting diarrhoea to severe diarrhoea, which can progress to pseudomembrane formation, toxic megacolon, perforation, shock and even death. During carriage or an infection, patients have the potential to release large amounts of spores into the environment (Jump *et al.*, 2007). In this way, the toxins and spores of *C. difficile* ensure a continual presence and spread in the human population.

TcdA and TcdB are encoded on the 19.6 kb pathogenicity locus (PaLoc), along with the positive regulator TcdR, the negative regulator TcdC and a putative holin, TcdE. Study of the five genes of the PaLoc has shown an increased transcription of *tcdA*, *tcdB*, *tcdR* and *tcdE* and a decreased transcription of *tcdC* during the progression of *C. difficile* from the exponential to the stationary phase of growth (Hundsberger *et al.*, 1997).

Since the early 2000s, ribotype 027 (BI/NAP1) has emerged as the cause of several outbreaks and disease of increased severity, morbidity and mortality (Loo *et al.*, 2005; Pépin *et al.*, 2005). It has been dubbed the hypervirulent strain, following the observation of excessive toxin production by this ribotype: up to 16 times more toxin A and 23 times more toxin B (Warny *et al.*, 2005). An explanation of this increased toxin production has been found in the deletions observed in the *tcdC* gene of most, but not all, ribotype 027 strains (MacCannell *et al.*, 2006; Spigaglia & Mastrantonio, 2002). The Δ117 frame-shift mutation results in a truncated protein lacking in

Abbreviations: CDI, *Clostridium difficile* infection; PaLoc, pathogenicity locus.

function. Strains with both Δ117 and an 18 bp deletion have also been found to produce more toxin in the exponential phase of growth and to cause more severe disease (Curry *et al.*, 2007). However, it has also been shown that this ribotype does not produce more toxin than others, although the duration of the toxin production is increased (Freeman *et al.*, 2007). Interestingly, it has been observed that 027 strains not only produce more toxins but also have increased sporulation rates, giving them an added advantage in dissemination (Akerlund *et al.*, 2008).

The changing epidemiology of *C. difficile* in Scotland has been studied extensively and has revealed changes in the ribotypes implicated in CDI over the years (Taori *et al.*, 2009). Ribotype 012, to which the reference and the first sequenced strain 630 belongs, represented 5 % of the *C. difficile* isolates collected between 1979 and 2004, but is no longer reported in the infected population. The incidence of ribotype 001 has increased over the years from 1.5 to 75.8 % (Mutlu *et al.*, 2007). Ribotype 106, not identified in Scottish isolates till 2004, represented 8.1 % of the isolates collected in 2005. In 2006, the prevalence of ribotype 001 started declining and that of ribotype 106 increased steadily. The same year saw the first case of ribotype 027 infection in Scotland, and its incidence has since been on the rise. The most common causes of CDI in Scotland today are ribotypes 106, 001 and 027 (Health Protection Scotland, 2008, 2010).

All the ribotypes mentioned above are multidrug-resistant and have been isolated from CDI patients. Though only ribotype 027 is considered to be hypervirulent, strains 106 and 001 have also been shown to cause severe disease (Arvand *et al.*, 2009; Sundram *et al.*, 2009). Strain 630 was isolated from a patient with pseudomembranous colitis during a diarrhoeal outbreak in a Zurich hospital (Wüst *et al.*, 1982). These observations led to the hypothesis that not only excessive toxin production but also enhanced growth and increased sporulation might explain the severity of disease caused by these strains, as well as their ability to spread in the environment. To test this, the growth of these strains over a 24 h period was studied, along with toxin production and sporulation. For a more detailed understanding, the transcription of the genes of the PaLoc and the sporulation initiator *spo0A* were investigated over the same time period. Strain VPI 10463 was used as the reference strain, as it is known to be a high toxin and low spore producer (Akerlund *et al.*, 2006).

METHODS

Bacterial strains. Three ribotypes of *C. difficile* were used in this study: 027 (a clinical isolate obtained from E. J. Kuijper, Leiden University Medical Center), 001 and 106 (isolates from toxin-positive faecal samples from CDI cases in south-east Scotland; Mutlu *et al.*, 2007). Strain 630 (ribotype 012, obtained from P. Mullany, UCL Eastman Dental Institute, London, UK) was used as a reference strain, representing a historic isolate. VPI 10463 (obtained from Unipath, Bedford, UK), a known high toxin producer, was the other reference strain. The strains were purified and maintained as spore suspensions in Robertson's cooked meat medium.

Growth measurement. A starter culture was prepared by inoculating 0.5 ml of the spore suspension into 3 ml pre-reduced anaerobic incubation medium (AIM) (Brown *et al.*, 1996). This was incubated anaerobically (80 % N_2, 10 % H_2, 10 % CO_2) for 16 h at 37 °C in a Mark III workstation (Don Whitley Scientific) till OD_{600} 1.0 ($\pm$0.05) was achieved. The starter culture (3 ml) was inoculated into 300 ml pre-reduced AIM. Growth was determined by measuring OD_{600} at 4, 8, 12, 16, 20 and 24 h. Cultures were checked for purity by Gram-staining and aerobic and anaerobic subculture on blood agar. All growth curves and related studies were performed in triplicate.

Total toxin production. Total toxin (A+B) production was measured using the *C. difficile* TOX A/B II kit (TechLab) according to the manufacturer's instructions. Culture supernatants were collected every 4 h by centrifugation at 13 000 ***g*** for 1 min and stored at −80 °C. The supernatants were diluted 1 : 5 in the supplied diluent and run in duplicate. Total toxin levels were determined by measuring A_{450}/OD_{600}.

ELISA for toxin A. A sandwich ELISA was developed for the quantification of toxin A. ELISA plates were coated with 50 µl of 1.5 µg ml^{-1} rabbit polyclonal antibody to *C. difficile* toxin A (Meridian Life Science) in 0.1 M carbonate/bicarbonate buffer (pH 9.6). The plates were incubated at 4 °C overnight. They were then washed with PBS (pH 7.4) containing 0.05 % Tween 20 and blocked with 100 µl PBS containing 3 % gelatin. Culture supernatants (50 µl) diluted in PBS with 1 % gelatin were then added to the plate in duplicate and incubated. To generate standard curves, twofold dilutions of toxin A (Calbiochem, Merck) from 250 to 0.25 ng ml^{-1} were added to each plate. The plates were washed as above and 50 µl of 0.5 µg ml^{-1} mouse monoclonal antibody to toxin A (Novus Biologicals) was added to the plate and incubated. After washing, 50 µl 1 : 1000 anti-mouse IgG (whole molecule)–peroxidase antibody produced in rabbits (Sigma) was added to the plates. All the above incubations were performed for 1 h at 37 °C. Finally, the plates were washed and 100 µl substrate, 3,3′,5,5′-tetramethylbenzidine (TMB), was added for 5 min at room temperature. The reaction was stopped with 100 µl 0.2 M H_2SO_4 and A_{450} was measured.

Quantitative cytotoxicity assay for toxin B. It proved impossible to develop an ELISA for toxin B with commercially available reagents, and thus a modified cytotoxicity assay was performed for its quantification. Vero cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10 % fetal bovine serum, 1 % non-essential amino acids, 100 units penicillin ml^{-1} and 100 µg streptomycin ml^{-1} at 37 °C in 5 % CO_2. The monolayers were trypsinized at 37 °C for 5 min, washed with medium and resuspended at 5×10^5 cells ml^{-1}. The cells were added to a 96-well tissue culture plate in 50 µl volumes and incubated overnight. The medium was replaced with 50 µl of suitable dilutions of culture supernatant prepared in medium, in duplicate. To generate standard curves, twofold dilutions of toxin B (Calbiochem, Merck) from 250 to 0.25 ng ml^{-1} were added to each plate. The plates were incubated for 48 h, washed with pre-warmed PBS and then 20 µl of 5 µg ml^{-1} thiazolyl blue tetrazolium bromide (Sigma) was added to the wells, followed by incubation at 37 °C for 4 h. Any formazan produced by the cells was dissolved in 100 µl DMSO (Sigma) and A_{570} was measured.

Real-time RT-PCR. Transcriptional analysis of the PaLoc genes and *spo0A* was performed. Culture was collected every 4 h corresponding to approximately 5×10^8 cells (50 ml at 4 h to 5 ml at 24 h) by centrifuging at 4000 ***g*** for 10 min. The pellets obtained were immediately treated with 500 µl RNAwiz (Ambion) or TRIzol (Invitrogen), vortexed vigorously in a Mini-BeadBeater (Biospec Products) and stored at −80 °C for a maximum of 7 days before use. RNA was extracted according to the manufacturer's instructions and treated with DNase I (Ambion) at 37 °C for 1 h, followed by deactivation of the DNase with DNase Inactivation Reagent (Ambion) for 2 min at room temperature. The quantity and quality of the RNA were assessed using a NanoDrop spectrophotometer, and aliquots were stored at −80 °C. The SuperScript VILO cDNA Synthesis kit (Invitrogen) was used to convert 2 µg RNA into cDNA according to the manufacturer's instructions. The cDNA was aliquoted and stored at −20 °C. Primers for *tcdA*, *tcdB*, *tcdC*, *tcdR*, *tcdE*, *spo0A* and *rrn* (16S rRNA gene) were designed using Primer3 software (Rozen & Skaletsky, 2000) based on the genome of strain 630 (Table 1). These were first tested by conventional PCR with genomic DNA to confirm specificity and product size. The real-time RT-PCRs were performed in duplicate in 20 µl volumes using 50 ng cDNA, primers at 200 nM for *tcdA–R*, 500 nM for *tcdE* and 100 nM for *spo0A* and *rrn*, and 10 µl SYBR Green JumpStart *Taq* ReadyMix (Sigma) in an Mx3000P quantitative PCR system (Stratagene). Standard curves were generated using fourfold dilutions of cDNA pools on each plate for each gene to determine the efficiency of the reactions. RNA and diethylpyrocarbonate (DEPC) water controls were also maintained. The thermal profile used was: initial denaturation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 20 s, annealing at 56 °C for 20 s, and extension at 72 °C for 20 s. This was followed by a dissociation curve to check the product specificity. The expression of the test genes was normalized to that of *rrn* and calculated by the Pfaffl method (Pfaffl, 2001) using the amplification efficiencies determined in each run. The 4 h value was used as the calibrator for expression at the successive time points.

Spore production. Spore production was assessed using 10 ml of the culture every 4 h from the same culture as above. Pellets were obtained by centrifugation at 4000 ***g*** for 10 min, washed twice in distilled water and treated with 50 % ethanol for 1 h. After washing twice, the pellets were suspended in 1 ml distilled water and 10-fold serial dilutions were plated onto blood agar. After anaerobic incubation for 48 h, the colonies were counted and the number of spores per millilitre of culture was determined.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 4.0 software. Strain comparison at individual time points was performed by one-way ANOVA. To assess the overall trends of growth and the corresponding phenotypic traits over the time-course, area under curve (AUC) analysis was performed for each strain, and strains were then compared by one-way ANOVA.

Table 1. Primer pairs used to amplify the genes studied by real-time RT-PCR

Gene	Primer sequence
tcdA	5′-GCTATTGCTGCAGTCGGATT-3′
	3′-TACCATTAACAGTCTGCCAACC-5′
tcdB	5′-TGGTGAAGATGGTGTCATGC-3′
	3′-TTCTCCCTCAAAATTCTCATCC-5′
tcdC	5′-TTTAAGAGCACAAAGGGTATTGC-3′
	3′-TGACCTCCTCATGGTCTTCAG-5′
tcdR	5′-AACTCAGTAGATGATTTGCAAGAA-3′
	3′-TTAAATCTGTTTCTCCCTCTTCA-5′
tcdE	5′-AAATATGTGCTTATGTGGATTACCAG-3′
	3′-TTCATCCTTAGCATTCATTTCATC-5′
spo0A	5′-TGTTGAGCTTTTAGGTGCAG-3′
	3′-TGCATGTCTTATTGCTCTTTCAA-5′
rrn	5′-AGTGAAAGGCTACGGCTCAA-3′
	3′-CTACGCATTTCACCGCTACA-5′

RESULTS AND DISCUSSION

The hypervirulence of ribotype 027 has been of increasing interest since the emergence of this strain and its apparent ability to cause severe disease and be responsible for many outbreaks (Kuijper *et al.*, 2007; Pépin *et al.*, 2004; Smith, 2005). The hypervirulence has been directly associated with the excessive toxin production observed in this ribotype. Here we show that ribotype 027 produces much greater amounts of toxin than other strains in this study. Also, ribotypes 106 and 001 produce more toxins than strain 630, and ribotype 106 produces markedly more spores than the other strains.

All the *C. difficile* strains show similar patterns of growth

The growth curves obtained for all the *C. difficile* strains were similar (Fig. 1). Ribotypes 001 and 106 showed slightly but significantly increased growth at 4 h when compared with strains 630 and VPI 10463 ($P<0.001$), but not when compared with ribotype 027. Throughout the 24 h, the growth of all the strains was

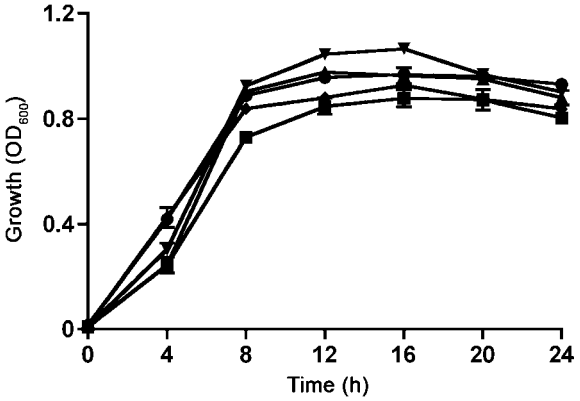

Fig. 1. Growth curves of five *C. difficile* strains. The growth of strain 630 (■), VPI 10463 (▲), ribotype 027 (▼), ribotype 001 (◆) and ribotype 106 (●) was measured by OD_{600} over 24 h. The patterns of growth were similar for all the strains. Error bars, SEM of 12 growth curves (performed in triplicate on four different occasions).

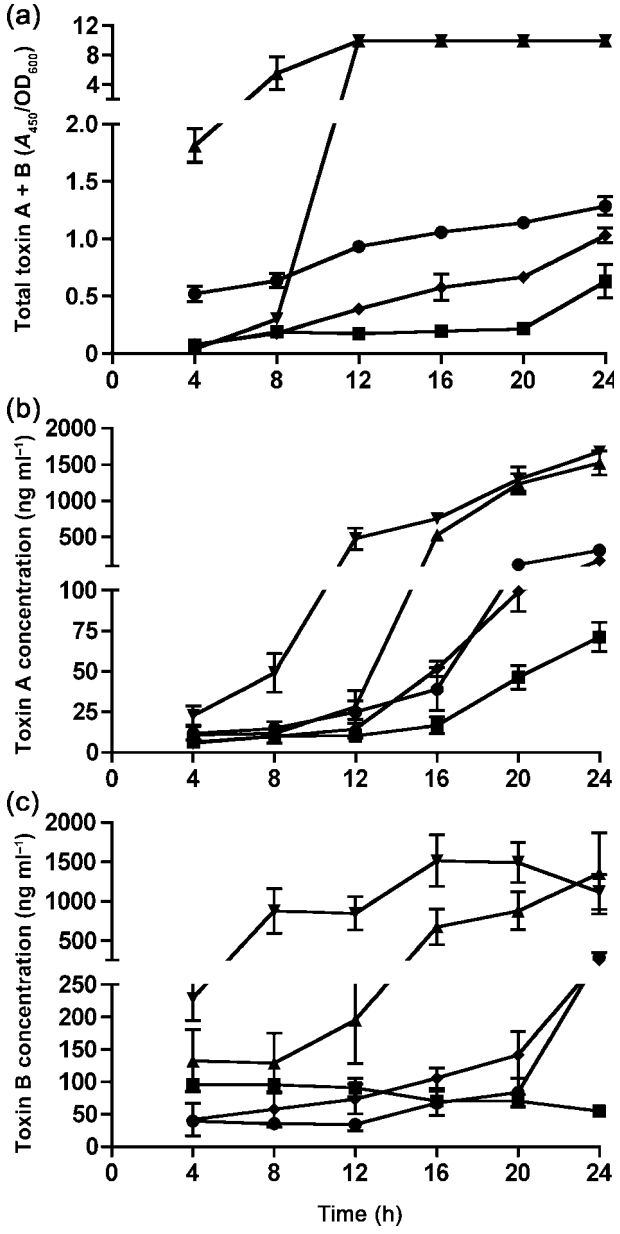

Fig. 2. Toxin production in five *C. difficile* strains. Toxin production in strain 630 (■), VPI 10463 (▲), ribotype 027 (▼), ribotype 001 (◆) and ribotype 106 (●) was measured over the 24 h period studied. (a) Total toxin production (A+B) measured using a combined kit showed significantly higher toxin production in ribotype 027 and VPI 10463. Error bars, SEM of three experiments. (b) Toxin A was quantified by a newly developed in-house ELISA. In all the strains, extracellular levels of toxin A increased over 24 h. Ribotype 027 produced significantly greater amounts of toxin A from 8 h. By the stationary phase, ribotype 027 and VPI 10463 produced markedly more toxin than ribotypes 106 and 001. Strain 630 produced the least toxin A. Error bars, SEM of six experiments. Lower *y* axis, 0–100 ng ml^{-1}; upper *y* axis, 100–2000 ng ml^{-1}. (c) Toxin B was quantified by a modified cytotoxicity assay. Ribotype 027 produced significantly greater amounts of toxin B from 8 h, while that in VPI 10463 increased steadily over time. In ribotypes 106 and 001, the amounts of toxin B increased gradually till 20 h and then showed a sudden increase at 24 h. In strain 630, toxin B levels decreased over time. Error bars, SEM of six experiments. Lower *y* axis, 0–250 ng ml^{-1}; upper *y* axis, 250–2000 ng ml^{-1}.

significantly higher than that of strain 630 ($P<0.001$). Thus, varying growth rates do not appear to explain the degrees of virulence observed in different strains of *C. difficile*.

C. difficile ribotype 027 produces significantly more toxin than other strains

Total toxin (A+B) production as measured by the combined ELISA kit varied significantly between the strains studied (Fig. 2a). In ribotype 027, total toxin production increased significantly between 8 and 12 h. By 12 h, ribotype 027 and VPI 10463 produced significantly more toxin than the other strains ($P<0.001$), which showed a gradual increase in toxin production over time. Ribotype 106 produced more toxin than 001, which in turn was greater than strain 630. Over the 24 h, ribotype 027 and VPI 10463 produced significantly higher amounts of total toxin ($P<0.001$). Beyond 12 h, the levels of toxin production in ribotype 027 and VPI 10463 could not be determined due to saturation of the assay. Thus, to investigate further the amounts of individual toxins produced by the different strains, quantitative methods were developed to detect each of the toxins.

Toxin A was detected in the cultures of all strains by ELISA (Fig. 2b). Ribotype 027 produced the most toxin A up to 12 h ($P<0.001$) and the amounts increased till 24 h. All the other strains produced low levels of toxin A till 12 h. Beyond 12 h, large amounts of toxin A were detected in cultures of VPI 10463, as expected, and the amounts were similar to those of ribotype 027. Ribotypes 106 and 001 produced toxin A at similar levels, which were significantly lower than those of VPI 10463 and ribotype 027 ($P<0.001$) and slightly higher than those of strain 630. Toxin B production was assessed using a modified cytotoxicity assay, and levels were marginally higher than those of toxin A in all strains over time (Fig. 2c). VPI 10463 showed a steady increase in toxin B production that reached high levels at 24 h. Ribotype 027 produced significantly more toxin B than the other strains at 8 h ($P<0.01$) and this level remained almost constant till 24 h. In ribotypes 106 and 001, toxin B production increased gradually up to 20 h and then increased sharply at 24 h, possibly due to accumulation in the culture medium. Interestingly, in strain 630, toxin B production decreased over time. Overall, toxin B production in the hypervirulent ribotype 027 was significantly higher than that in the other test strains ($P<0.01$).

From these results, it is evident that ribotype 027 produces significantly more toxin than the other strains. As

observed by others, our data also suggested that ribotype 027 is capable of producing up to 20 times more toxin (A or B) than other strains (Warny *et al.*, 2005), except VPI 10463. This was also true for seven isolates of ribotype 027 from Scotland and five from the Netherlands (data not shown) (Vohra & Poxton, 2010). This appears to be a phenotypic advantage for this strain, enabling it to cause severe disease.

Another observation was the detection of moderately more toxin A than toxin B at 24 h in ribotypes 027 and 106 (Fig. 4). Toxin A, an enterotoxin with the ability to bind to epithelial cells in the gut via receptors (Krivan *et al.*, 1986; Tucker & Wilkins, 1991), causes initial damage by glucosylation of Rho proteins (Aktories *et al.*, 2000). In animal studies, it has been observed that in the absence of toxin A, toxin B is unable to induce the pathology characteristic of CDI (Lyerly *et al.*, 1985). Even in cell cultures with Caco2 cells, it has been demonstrated that when toxin A challenge is removed, no damage is caused to the epithelial barrier (Sutton *et al.*, 2008). Thus, large amounts of toxin A may contribute to increased disease severity and perhaps the increased potential of ribotypes 027, 106 and 001 to induce CDI in healthy humans: the greater the initial damage to the gut by toxin A, the greater the chance of toxin B causing extensive cytotoxicity. However, outbreaks caused by A^-B^+ strains have been reported (Drudy *et al.*, 2007), questioning the clinical importance of toxin A in disease. Toxin B is 1000-fold more cytotoxic than toxin A; however, its role in the development of the characteristic pathology of CDI is debated, though A^+B^- strains have not yet been isolated. It has also been shown that toxin B knockout strains are ineffective at causing fatal disease in hamsters (Lyras *et al.*, 2009). However, more recently it has been suggested that both toxins are important in CDI (Kuehne *et al.*, 2010). Whatever the importance of toxin B in disease, it is clear that the current epidemic strains produce large quantities of it. This, coupled with the production of large amounts of toxin A, could explain the severity of disease associated with ribotypes 027, 106 and 001.

High toxin producers show increased transcription of the PaLoc genes

Transcriptional analysis of the PaLoc genes was performed by real-time RT-PCR (Fig. 3). In VPI 10463, the transcription of *tcdA*, *tcdB* and *tcdR* increased over 24 h, while that of *tcdC* decreased after 8 h, as shown by Hundsberger *et al.* (1997). *tcdE* levels were found to increase till 12 h, after which they decreased, with a transient increase at 24 h. This served as a basis for transcription studies in the other strains using the methodology developed. However, varying patterns of PaLoc gene expression were observed in the other strains.

tcdA expression increased till 12 h in ribotype 027, ribotype 106 and strain 630, and then decreased, whilst remaining almost constant in ribotype 001 over time. A similar trend was observed for *tcdB* in ribotype 106 and strain 630, though in the latter, the levels were below the 4 h value. In ribotype 001, an increase in *tcdB* transcription was observed at 20 h. Interestingly, ribotype 027 was the only strain to show constantly increasing *tcdB* expression over the 24 h period studied, though it was less than that of *tcdA*. The pattern of *tcdA* expression suggests that transcription peaks at 12 h and the toxin levels detected in culture thereafter are the result of accumulation. This was observed less markedly and from 8 h for toxin B. This observed correlation between gene expression and toxin detection (Fig. 4) was analysed by expressing the results as the ratio of toxin production to toxin gene transcription. Though the transcription of *tcdB* was lower than that of *tcdA* in all the strains, the levels of toxin B detected were always higher than those of toxin A, suggesting more efficient production of TcdB or perhaps greater degradation of TcdA in culture.

tcdR transcription increased steadily over time in strain 630, but in ribotypes 027, 001 and 106, peak expression was observed at 12 h, followed by a decline. A transient increase in expression was observed at 24 h in ribotypes 027 and 106. Notably, *tcdC* transcription showed a similar pattern and at similar levels, contrary to the observations in VPI 10463.

tcdC is the negative regulator of toxin production (Dupuy *et al.*, 2008; Matamouros *et al.*, 2007), and it has been shown that its transcription decreases as cultures enter stationary phase (Hundsberger *et al.*, 1997). The levels of the protein itself have also been shown to diminish over time (Govind *et al.*, 2006). Further, the deletions found in *tcdC* in ribotype 027 have been used to explain the excessive toxin production in this strain, as the truncated TcdC protein would be ineffective in preventing the complexing of TcdR with RNA polymerase (Curry *et al.*, 2007). Contrary to the gene and protein studies, our data showed that *tcdC* transcription increased over time, at least till 12 h, and then decreased. Though there was an evident decrease in *tcdC* expression, it was not considered to fit into the pattern described elsewhere (Hundsberger *et al.*, 1997), as the *tcdR* expression observed showed a similar pattern and was at similar levels. This suggests that *tcdC* might have a modulatory effect on toxin production, rather than a strictly inhibitory one. Also, both toxins, A and B, were detected in the exponential and stationary phases of growth, despite increasing *tcdC* expression. Others have also shown this expression of *tcdC* in both the phases of growth, though slightly diminished in the stationary phase (Dineen *et al.*, 2007; Karlsson *et al.*, 2008). It is possible that TcdC, being a membrane-associated protein, has an effect on the release of toxins. These hypotheses could be tested by gene knockout and protein interaction studies, and would greatly add to the understanding of toxin production and release in *C. difficile*.

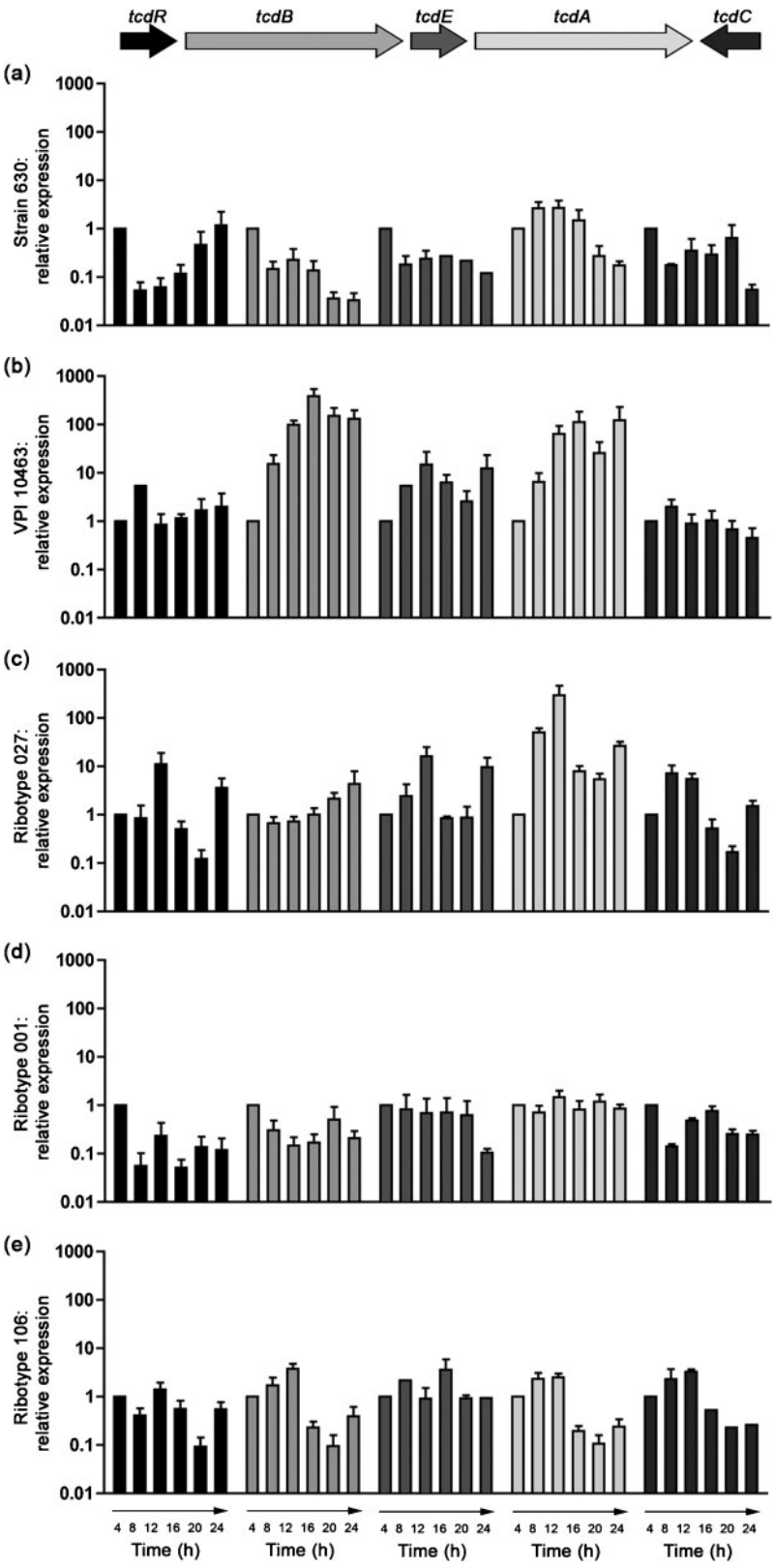

Fig. 3. Transcription of the PaLoc in five *C. difficile* strains. Transcription of the PaLoc genes was studied by real-time RT-PCR in (a) strain 630, (b) VPI 10463, (c) ribotype 027, (d) ribotype 001 and (e) ribotype 106. The expression of all the genes was normalized to that of *rrn*, using the 4 h value as the baseline of expression. (a) In strain 630, *tcdA* expression increased till 12 h and then decreased, similar to *tcdB*, though that of *tcdB* was below the 4 h value. *tcdR* and *tcdC* expression increased over time, while that of *tcdE* remained constant. (b) In VPI 10463, the expression of *tcdR*, *tcdA*, *tcdB* and *tcdE* increased over the 24 h, while that of *tcdC* decreased. (c) In ribotype 027, the transcription of *tcdR*, *tcdE*, *tcdA* and *tcdC* peaked at 12 h and then decreased. *tcdB* transcription increased over 24 h. (d) In ribotype 001, *tcdR* and *tcdC* transcription was similar, while that of *tcdB*, *tcdE* and *tcdA* did not vary considerably over time. (e) In ribotype 106, *tcdR*, *tcdB*, *tcdA* and *tcdC* transcription peaked at 12 h, while that of *tcdE* increased till 16 h. Thereafter, expression of all the genes decreased. Error bars, SEM of six experiments for *tcdA* and *tcdB* expression, and four experiments for *tcdR*, *tcdE* and *tcdC* expression.

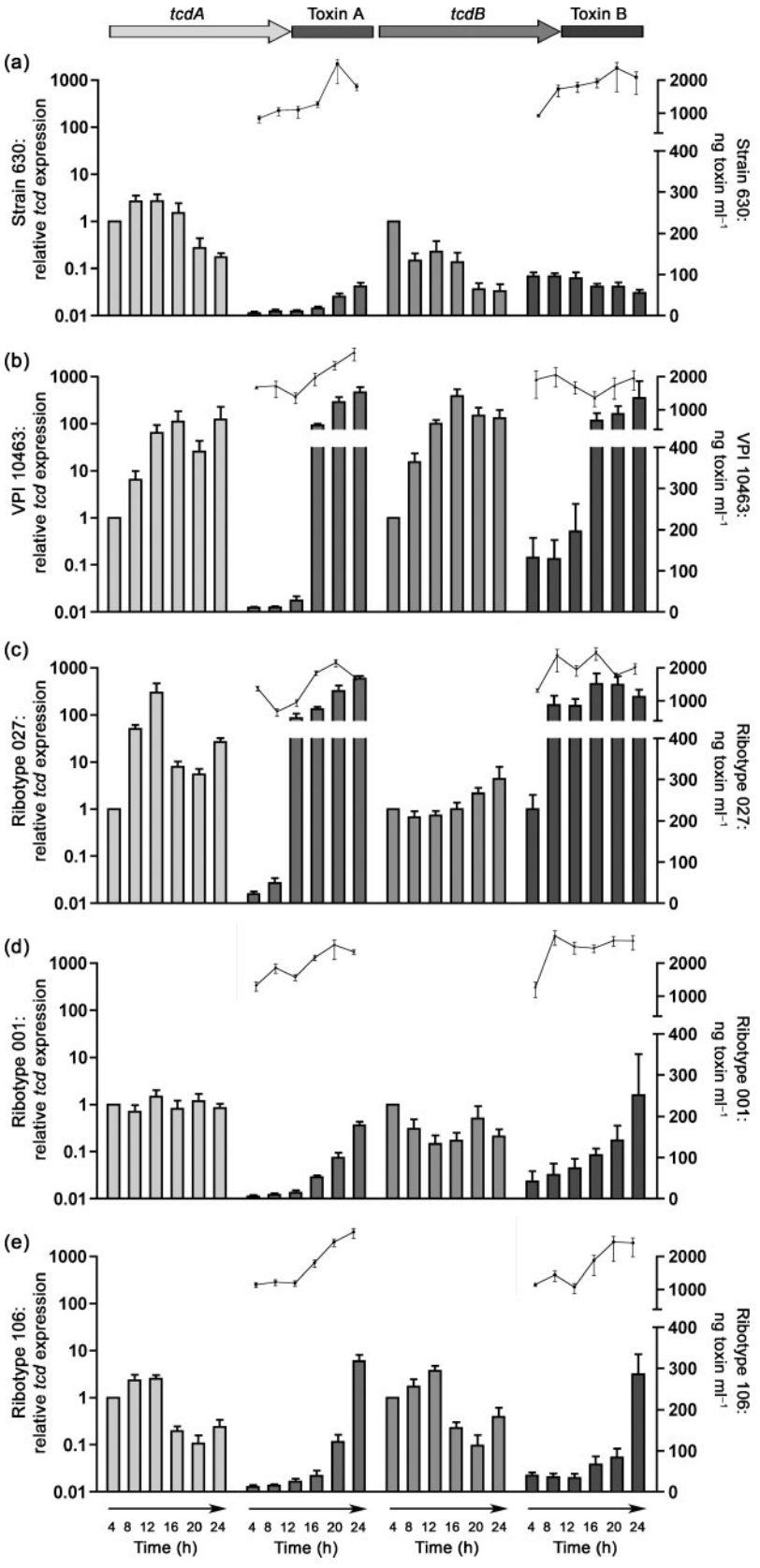

Fig. 4. *tcdA* and *tcdB* transcription and the corresponding toxin A and toxin B production. The transcription of the toxin genes *tcdA* and *tcdB* and the corresponding production of toxin A and toxin B was studied in (a) strain 630, (b) VPI 10463, (c) ribotype 027, (d) ribotype 001 and (e) ribotype 106. The extracellular levels of both toxins increased over time in all the strains, even when gene expression decreased. Ribotype 027 produced greater amounts of both toxins and also showed greater gene expression. The correlation of toxin production to toxin gene transcription was assessed by analysing ratios of toxin A value : *tcdA* expression and toxin B value : *tcdB* expression at the different time points. This is represented by the line graphs above the toxin values for each toxin ($\log_{10}$ scale ranging from 10^{-1} to 10^{5}). In all the strains, the release of toxin A at 12 h is evident. For toxin B, earlier release is indicated. Error bars, SEM of six experiments.

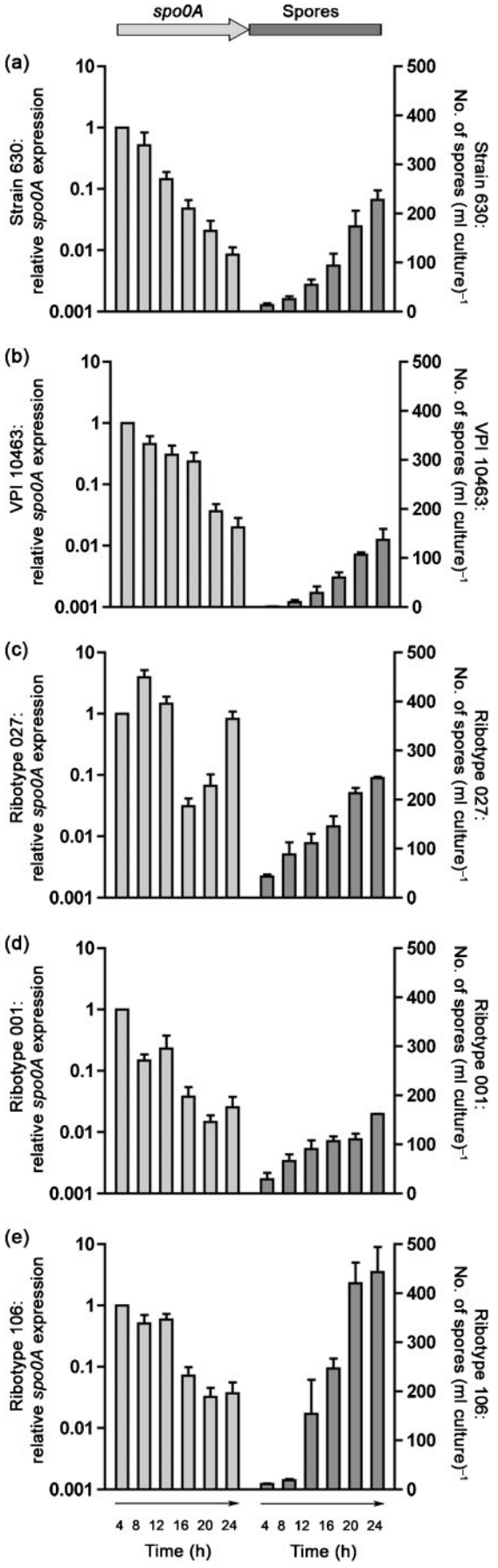

Fig. 5. *spo0A* transcription and spore production in five *C. difficile* strains. *spo0A* transcription, normalized to that of *rrn*, in (a) strain 630, (b) VPI 10463, (c) ribotype 027, (d) ribotype 001 and (e) ribotype 106 decreased over time, except for the slight increase in ribotype 027 at 8 and 12 h. The number of spores detected in the cultures of all the strains increased over the 24 h, with ribotype 106 producing the highest number of spores, followed by ribotypes 027 and 001 and strain 630. VPI 10463 produced the lowest number of spores. Error bars, SEM of six experiments for the transcription of *spo0A* and three experiments for spore production.

tcdE transcription was also studied, though not in a very efficient reaction. However, the results obtained showed that there was a major increase, approximately 15-fold, in *tcdE* expression till 12 h in ribotype 027 and VPI 10463, the highest toxin producers, which corresponds to the time beyond which large amounts of toxin are detected in the cultures of these strains. The almost steady expression of *tcdE* in the other strains corresponded to the slower release of toxins seen in those strains. Thus, it is likely that TcdE plays an important role in the release of the *C. difficile* toxins, given its holin-like properties (Tan *et al.*, 2001). The increased *tcdE* transcription in ribotype 027 may also contribute to hypervirulence.

Currently common *C. difficile* ribotypes produce more spores than a previously dominant strain

Sporulation is the other key trait of *C. difficile* that enables it to survive and spread in the environment. That sporulation and toxin production are alternate mechanisms for survival has been debated (Kamiya *et al.*, 1992). VPI 10463 fits this idea, showing high toxin production and low sporulation. Contrary to this, ribotype 027 has been shown to produce high levels of both toxins and spores, and this has been observed in 12 different isolates of ribotype 027 (data not shown) (Vohra & Poxton, 2010).

All the strains studied here produced alcohol-resistant spores, and their numbers in culture increased over time (Fig. 5). Ribotype 106 produced significantly more spores ($P<0.001$) than the other strains, though this significance was less when compared with spore production by ribotype 027 ($P<0.05$). At 24 h, a final count of 443 spores (ml culture)$^{-1}$ was obtained for ribotype 106, which was significantly higher than that of the other strains. VPI 10463 produced the fewest spores. The number of spores obtained was very low, although this was possibly due to the absence of bile salts, which enhance spore germination (Wilson, 1983), in the medium used.

spo0A, the master regulator of sporulation, was selected as the indicator of the magnitude of the spore-producing capacity of the *C. difficile* strains studied. *spo0A*

transcription in all the strains decreased over time (Fig. 5). In the epidemic strains, however, there was a slight difference in the pattern of expression: in ribotype 027, *spo0A* expression increased at 8 h before decreasing and then spiked at 24 h, whilst in ribotypes 106 and 001, the levels of expression increased marginally at 12 h before falling. These differences, however, were not significant.

Despite the expected decrease in gene expression, the trends over the first few hours suggested subtle inter-strain differences. Perhaps the increased duration of transcription of *spo0A* in the early stages of growth enhances the expression of the subsequent genes involved in the process, resulting in a greater number of spores being produced and released into the environment, irrespective of environmental stresses. The link between toxin and spore production has recently been described: *spo0A* mutants show decreased sporulation and toxin production (Underwood *et al.*, 2009). Thus, the observed increase in *spo0A* transcription in ribotype 027 might also increase its toxin production and add to its hypervirulence.

During the preparation of this manuscript, a similar study was published comparing hypervirulent ribotype 027 (BI) strains with non-hypervirulent strains (Merrigan *et al.*, 2010). The increase in the transcription of the PaLoc genes, including *tcdC*, was also observed in that study, and those authors too hypothesize a modulatory function for TcdC. However, unlike their results, we detected toxin production in all our strains in the exponential phase of growth as well as the stationary phase. It is possible that this difference was due to the medium used. We used anaerobic incubation medium (AIM) in our experiments, a medium that does not contain any glucose, which inhibits toxin production (Dupuy & Sonenshein, 1998; Karlsson *et al.*, 1999). It does contain cysteine, though at subinhibitory levels. This might explain the earlier detection of toxins in our study and the greater levels detected in the stationary phase of growth. This might also address the significantly higher *tcdA* transcription in ribotype 027 observed in our study.

Although a single isolate was tested for each ribotype, from the data presented here, we can conclude that ribotype 027 has the ability to produce large amounts of toxins and spores, both key phenotypic advantages that are likely to have aided its emergence. Further, ribotype 106 possesses traits that may directly address its presence as the strain most commonly associated with CDI in Scotland. The increased toxin and spore production in the current epidemic strains corresponds directly with the severity of disease and extent of spread associated with them, and gives further insight into the evolving bacterial factors that affect the epidemiology of CDI.

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Efficacy of decontaminants and disinfectants against *Clostridium difficile*

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Clostridium difficile is a common nosocomial pathogen transmitted mainly via its spores. These spores can remain viable on contaminated surfaces for several months and are resistant to most commonly used cleaning agents. Thus, effective decontamination of the environment is essential in preventing the transmission of *C. difficile* in health-care establishments. However, this emphasis on decontamination must also be extended to laboratories due to risk of exposure of staff to potentially virulent strains. Though few cases of laboratory-acquired infection have been reported, the threat of infection by *C. difficile* in the laboratory is real. Our aim was to test the efficacy of four disinfectants, Actichlor, MicroSol 3+, TriGene Advance and Virkon, and one laboratory decontaminant, Decon 90, against vegetative cells and spores of *C. difficile*. Five strains were selected for the study: the three most commonly encountered epidemic strains in Scotland, PCR ribotypes 106, 001 and 027, and control strains 630 and VPI 10463. MICs were determined by agar dilution and broth microdilution. All the agents tested inhibited the growth of vegetative cells of the selected strains at concentrations below the recommended working concentrations. Additionally, their effect on spores was determined by exposing the spores of these strains to different concentrations of the agents for different periods of time. For some of the agents, an exposure of 10 min was required for sporicidal activity. Further, only Actichlor was able to bring about a 3 $\log_{10}$ reduction in spore numbers under clean and dirty conditions. It was also the only agent that decontaminated different hard, non-porous surfaces artificially contaminated with *C. difficile* spores. However, this too required an exposure time of more than 2 min and up to 10 min. In conclusion, only the chlorine-releasing agent Actichlor was found to be suitable for the elimination of *C. difficile* spores from the environment, making it the agent of choice for the decontamination of laboratory surfaces.

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INTRODUCTION

Clostridium difficile is the causative agent of *C. difficile* infection (CDI) (Bartlett *et al.*, 1978; Bartlett, 2008; George *et al.*, 1978). Though CDI is a common nosocomial infection, it is not limited to the hospital environment; community-acquired CDI and asymptomatic carriage are also common (Freeman *et al.*, 2010).

C. difficile is acquired via the faecal–oral route as spores (Lyerly *et al.*, 1988). Symptomatic patients shed large amounts of both vegetative cells and spores into the environment (Wilcox *et al.*, 2003). Though vegetative cells survive under aerobic conditions for only 15 min on dry surfaces, they can survive for up to 6 h on moist surfaces (Weber *et al.*, 2010). The spores of *C. difficile*, however, can persist on hospital floors for up to 5 months (Kim *et al.*, 1981) and are resistant to several cleaning agents, especially in the presence of organic matter (Fawley *et al.*, 2007; Wheeldon *et al.*, 2008). Surfaces contaminated with *C. difficile* spores can facilitate cross-colonization (Fawley & Wilcox, 2001). It has been suggested that health-care workers (and patients) acquire *C. difficile* from contaminated surfaces (Gerding *et al.*, 1995; McFarland & Stamm, 1986) such as portable commodes, bedpans, blood pressure cuffs, walls, floors, washbasins and furniture (Fekety *et al.*, 1981; Samore *et al.*, 1996; Weber *et al.*, 2010). A correlation between transmission of *C. difficile* among patients and contamination of the hands of health-care workers has been clearly observed (McFarland *et al.*, 1989).

Although *C. difficile* is a dreaded nosocomial pathogen, the risk of laboratory-acquired CDI has not been given much attention, in spite of the exposure of laboratory workers to relatively high inocula of *C. difficile*. However, cases of CDI have been reported in laboratory personnel (Bouza *et al.*, 2008) as well as in health-care workers (Arfons *et al.*, 2005; Hell *et al.*, 2009; Ray & Donskey, 2003). Thus, it is important to ensure proper decontamination of surfaces in the laboratory as well as proper hand-washing technique to

Abbreviation: CDI, *Clostridium difficile* infection.

Table 1. Agents used in this study

Agent	Active ingredient(s)	Manufacturer	Recommended working concentration
Actichlor	Sodium dichloroisocyanurate	Ecolab	1000 p.p.m. chlorine
Decon 90	Anionic and non-anionic surfactants	Decon Laboratories	1 : 10 dilution
Microsol 3+	Tertiary alylamine and quaternary ammonium compounds	Anachem	1 : 10 dilution
TriGene Advance	Polymeric biguanide hydrochloride and organic quaternary compounds	Medichem International	1 : 100 dilution
Virkon	Potassium peroxymonosulfate	Antec International	1 : 100 dilution

prevent potential transmission of *C. difficile* from the laboratory environment into the community and hospitals. The aim of this study was to test the efficacy of five commonly used agents against both vegetative cells and spores of *C. difficile* using laboratory tests as well as a more real-life surface cleaning approach.

METHODS

Bacterial strains. Five strains of *C. difficile* were used in this study: strain 630 (ribotype 012, obtained from P. Mullany, London, UK), strain VPI 10463 (obtained from Unipath, Bedford, UK), ribotype 027 (obtained from E. J. Kuijper, Leiden, The Netherlands), ribotype 001 and ribotype 106 (local strains from Edinburgh, UK). The strains were purified and maintained as spore suspensions in Robertson's cooked meat medium (Oxoid). All culturing of strains was performed anaerobically (80 % H_2, 10 % N_2, 10 % CO_2) at 37 °C in a Mark III workstation (Don Whitley Scientific).

Agents. Five agents were tested in this study: Actichlor, a disinfectant routinely used in hospitals; Decon 90, a laboratory decontaminant; and three commonly used laboratory disinfectants, Microsol 3+, TriGene Advance and Virkon. The active ingredients, recommended concentrations and manufacturers are listed in Table 1.

MIC testing. MICs of the agents for the vegetative cells of the different *C. difficile* strains were determined by the Wadsworth agar dilution method (NCCLS, 2004). Strains were cultured anaerobically on blood agar at 37 °C for 24 h, followed by culturing in pre-reduced thioglycollate broth supplemented with 5 μg haemin ml^{-1} and 1 μg vitamin K_1 ml^{-1} for 8 h. This ensured that the cultures were in the exponential phase when used for MIC testing, and thus consisted of mainly vegetative cells and very few spores. These cultures were diluted to a 0.5 McFarland standard for inoculation onto pre-reduced *Brucella* agar (Oxoid) supplemented with 5 % defibrinated horse blood, 5 μg haemin ml^{-1} and 1 μg vitamin K_1 ml^{-1} containing doubling dilutions of one of the five agents ranging from the manufacturer's recommended working concentration to 1/1024 of the same. The plates were incubated anaerobically at 37 °C for 48 h and then examined for growth. The lowest concentration of the tested agent to inhibit visible bacterial growth was recorded as the MIC of that agent. The experiments were performed in triplicate.

MICs were also performed by broth microdilution in a 96-well plate. Doubling dilutions of the five agents ranging from the manufacturer's recommended working concentration to 1/1024 of the same were prepared in pre-reduced anaerobic incubation medium and 100 μl of each dilution was added to wells of the plate in duplicate. To the wells, 10 μl culture adjusted to a 0.5 McFarland standard was added. The plates were incubated anaerobically at 37 °C for 48 h and then examined for growth by eye and by measurement of OD_{600}. Negative controls with no added culture and positive controls without the agents were also maintained in duplicate. The lowest concentration of the tested agent to inhibit visible bacterial growth and OD_{600} $\geqslant 0.1$ was recorded as the MIC of that agent. The experiments were performed in triplicate.

Preparation of spores. Strains were cultured in 500 ml pre-reduced anaerobic incubation medium (Brown *et al.*, 1996) anaerobically at 37 °C for 7 days. The cultures were then collected by centrifugation at 4000 ***g*** for 10 min. The pellets obtained were washed twice with sterile PBS and then treated with 50 % ethanol for 1 h with constant shaking. The pellets were collected by centrifugation, washed twice in PBS and then resuspended in 1 ml sterile distilled water and stored at 4 °C. Before each experiment, spore counts were determined from 10-fold serial dilutions of the final spore suspension plated on blood agar and incubated for 48 h.

Sporicidal assays. Spore preparations were standardized to 10^6 spores ml^{-1}. The agents were tested for their sporicidal activity in a

Table 2. MIC of each agent for vegetative cells of five *C. difficile* strains represented as a fraction of the recommended working concentration

Values are the median from both the agar-dilution and the broth microdilution methods for MIC testing.

Agent	Strain 630	Strain VPI 10463	Ribotype 027	Ribotype 001	Ribotype 106
Actichlor	1/8	1/8	1/2	1/8	1/8
Decon 90	1/4	1/4	1/2	1/2	1/4
Microsol 3+	1/128	1/128	1/128	1/128	1/128
TriGene Advance	1/32	1/32	1/32	1/32	1/32
Virkon	1/8	1/8	1/4	1/4	1/8

Table 3. Minimum sporicidal concentration of the agents for five *C. difficile* strains represented as a fraction of the recommended working concentration after different times of exposure

The tests were performed in 1 ml volumes with 10^5 spores being exposed to the disinfectants.

Agent*	Time of exposure (min)	Minimum sporicidal concentration				
		Strain 630	Strain VPI 10463	Ribotype 027	Ribotype 001	Ribotype 106
Actichlor	2	<1/5	<1/5	1/2	<1/5	1/2
	10	<1/5	<1/5	<1/5	<1/5	<1/5
	30	<1/5	<1/5	<1/5	<1/5	<1/5
Decon 90	2	1	1/2	1/2	1	1/2
	10	1/2	<1/5	<1/5	1/2	<1/5
	30	<1/5	<1/5	<1/5	<1/5	<1/5
Virkon	2	1	1	1	1	1
	10	1	<1/5	1	<1/5	<1/5
	30	1	<1/5	1	<1/5	<1/5

*The minimum sporicidal concentration for Microsol 3+ and TriGene Advance for all the strains was less than 1/5 at 2, 10 and 30 min.

suspension test at the recommended working concentration and 1/2 and 1/5 of the same. For each test, 100 µl spore suspension containing 10^5 spores was added to 900 µl of each of the dilutions and mixed thoroughly. At 2 min, 10 min and 30 min, 100 µl of the test was inoculated into 900 µl pre-reduced anaerobic incubation medium, mixed and incubated at 37 °C for 5 days in duplicate. Positive controls without any disinfectant and negative controls without spores were also maintained in duplicate to check that the medium was sustaining the germination of spores and growth of the resulting vegetative cells and that it was not contaminated, respectively. The tubes were examined for growth and the lowest concentration of the agent showing no growth was recorded as the minimum sporicidal concentration. Samples from tubes with no visible growth were plated to identify cidal or static activity and these were examined for growth after 5 days of anaerobic incubation.

Determination of $\log_{10}$ reduction. To determine the $\log_{10}$ reduction in *C. difficile* spores in suspension tests, spores were treated with the cleaning agents at the recommended working concentration in the presence or absence of organic matter. For the tests in the absence of organic matter, 100 µl spores in distilled water (approx. 10^4 spores ml^{-1}) was added to 900 µl agent. After 10 min, the spores were collected by centrifugation at 16 000 ***g*** for 2 min. The spore pellets were washed twice with 1 ml distilled water. The spores were then resuspended in 1 ml distilled water and 100 µl of this suspension was spread onto blood agar plates in duplicate and incubated at 37 °C anaerobically for 48 h. Untreated positive controls were maintained in duplicate. For the tests in the presence of organic matter, BSA (Sigma) was introduced into the test at a final concentration of 0.27 %. The $\log_{10}$ reduction was calculated as $\log_{10}(N_0/N_{10})$, where N_0=the number of spores in the positive control and N_{10}=the number of viable spores recovered from the test at 10 min.

Surface decontamination testing. Five different surfaces were used in this study: aluminium, glass, plastic, self-adhesive vinyl tiles and white ceramic tiles. The surfaces were autoclaved and dried in a hot air oven before use: the self-adhesive vinyl tiles could not be autoclaved and were cleaned with 70 % ethanol before use. On all the clean surfaces, squares of approximately 1 cm^2 were marked out with a wax crayon. These were then artificially contaminated with spores of the five *C. difficile* strains and tested for cleaning efficiency with all the cleaning agents. Each square was contaminated with 10 µl spore suspension (10^5 spores ml^{-1}) and left to air dry for 2 h. Fifty microlitres of cleaning agent at the manufacturer's recommended concentration was then added to the square on top of the dried spore suspension. After 2 min and 10 min, the area was scratched with a pipette tip 10 times in an attempt to mimic scrubbing and the agent was aspirated. The area was then washed with 100 µl distilled water. The aspirated agent and wash were added to 850 µl distilled water to obtain a final volume of 1 ml. One hundred microlitres of this was plated on blood agar and incubated anaerobically for 48 h. The surfaces were also cleaned only with water and these served as positive

Table 4. Mean $\log_{10}$ reduction in *C. difficile* spores of five strains in the absence and presence of organic matter

The $\log_{10}$ reduction is a mean of the values obtained for all five *C. difficile* strains.

Agent	$\log_{10}$ reduction (mean ± SEM)	
	Organic matter negative	Organic matter positive
Actichlor	3.093 ± 0.2239	3.076 ± 0.2429
Decon 90	0.5159 ± 0.06722	0.3384 ± 0.101
Microsol 3+	1.519 ± 0.1025	0.7288 ± 0.1294
TriGene Advance	1.698 ± 0.0806	0.5399 ± 0.09384
Virkon	1.171 ± 0.1705	0.1657 ± 0.04113

controls. The $\log_{10}$ reduction was calculated as above from two independent experiments.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 4.0 software. In order to compare agents and strains, one-way analysis of variance (ANOVA) was performed.

RESULTS AND DISCUSSION

Efficacy against vegetative cells

The growth of vegetative *C. difficile* was effectively suppressed by all the agents tested. The MICs of all agents for the strains were found to be the same by agar dilution and broth microdilution and these values were lower than the manufacturer's recommended concentration (Table 2). However, for Actichlor, Decon 90 and Virkon, higher concentrations seemed to be required to destroy vegetative cells of the epidemic ribotypes 027, 001 and 106. Microsol 3+ appeared to be the most effective agent, active at a 128-fold dilution of the recommended concentration, followed by TriGene, which was effective at a 32-fold dilution of the recommended concentration. Actichlor, used for routinely cleaning hospitals, was effective at its recommended concentration, but less than a 2-fold dilution was unable to inhibit the growth of the hypervirulent ribotype 027. These observations suggest that the epidemic ribotypes may have a greater resistance to commonly used cleaning agents. Also, they emphasize the importance of preparing these agents at the correct concentration.

Efficacy against spores

The agents tested were also found to be sporicidal at the recommended concentration in suspension tests at 2, 10 and 30 min of testing (Table 3). Once again it was observed that ribotype 027 required a greater concentration of Actichlor for destruction of its spores. This was true for the epidemic ribotype 106 as well. For Virkon, any dilution below the recommended concentration was not sporicidal at 2 min, making it a less effective disinfectant than Actichlor. Interestingly, Microsol 3+ and TriGene Advance were found to be sporicidal at a fivefold dilution of the recommended concentration even at 2 min of exposure. However, a neutralizer was not used in these experiments, and the agents were not removed by washing: they were diluted 10-fold into the recovery medium. Thus, it is possible that there was a prolonged exposure of the spores to low but effective concentrations of the agents tested.

Effect of organic matter on efficacy

All the selected agents except Decon 90 are marketed as sporicidal agents. Thus, it was of interest to determine the $\log_{10}$ reduction in spore numbers brought about by them at the recommended concentration. Under clean conditions, Actichlor was found to be the most effective

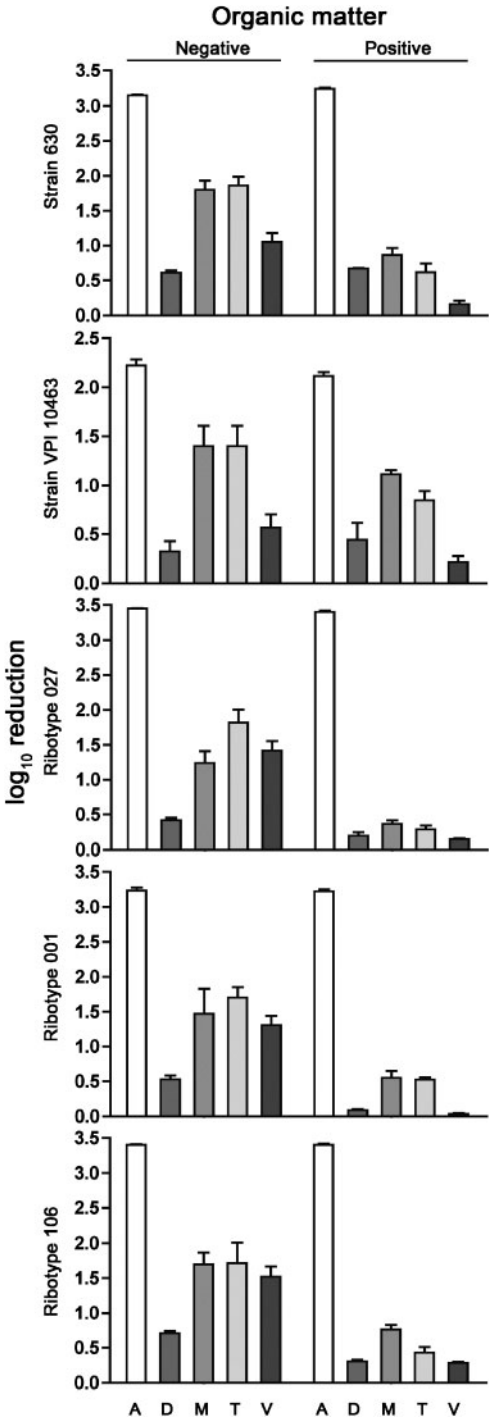

Fig. 1. Efficacy of Actichlor (A), Decon 90 (D), Microsol 3+ (M), TriGene Advance (T) and Virkon (V) ($\log_{10}$ reduction) against the spores of five strains of *C. difficile* in the absence and presence of organic matter. Only Actichlor effectively destroyed spores of all the strains in the absence or presence of organic matter, causing 3 $\log_{10}$ reduction in spores. The efficacy of all the other agents decreased significantly in the presence of organic matter, especially in destroying spores of the epidemic ribotypes 027, 001 and 106. Bars indicate ± SEM of two experiments.

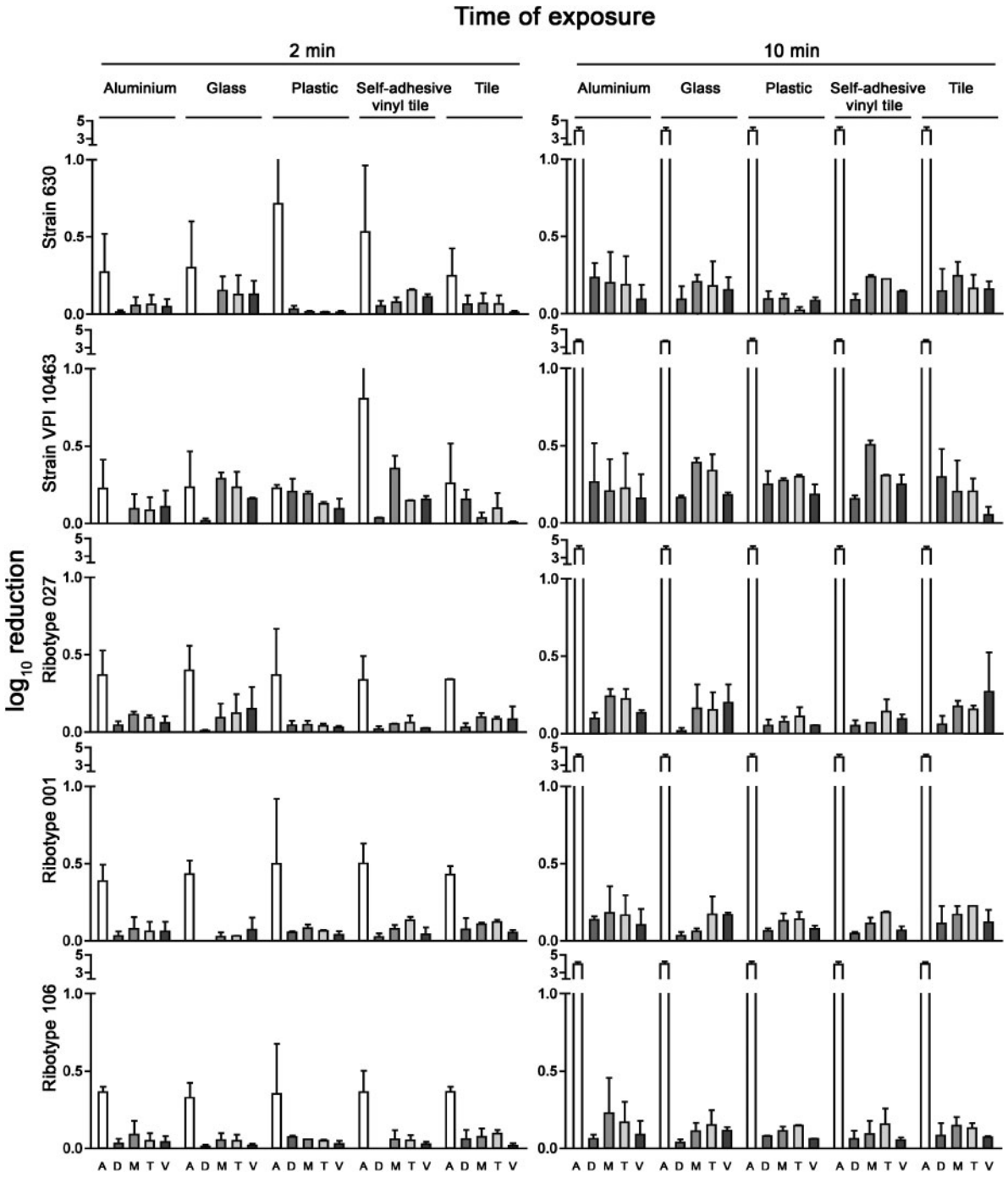

Fig. 2. Decontamination of different surfaces contaminated with spores of five *C. difficile* strains by Actichlor (A), Decon 90 (D), Microsol 3+ (M), TriGene Advance (T) and Virkon (V) tested at 2 min and 10 min of exposure. Actichlor (A) was the most effective decontaminant at both the time points and destroyed 100 % of spores of all the strains by 10 min. The other agents fared poorly, especially against the epidemic ribotypes 027, 001 and 106. Bars indicate ± SEM of two experiments.

sporicidal agent ($P<0.001$), bringing about a 3 $\log_{10}$ reduction of *C. difficile* spores at 10 min, at which time Microsol 3+, TriGene Advance and Virkon only caused approximately 1.5, 1.7 and 1.2 $\log_{10}$ reduction (Table 4). However, these disinfectants were still significantly more effective than Decon 90 ($P<0.001$ compared to Microsol 3+ and TriGene Advance; $P<0.01$ compared to Virkon). The superior efficacy of chlorine-releasing agents as compared to peroxy compounds against the spores of *C. difficile* has been observed previously (Lawley *et al.*, 2010).

No inter-strain differences were observed, except when Decon 90 was used; the least $\log_{10}$ reduction was observed for ribotype 027 ($P<0.05$). In the presence of organic matter, the efficacy of all the cleaning agents, except Actichlor, dropped considerably. Actichlor was the most effective disinfectant even in the presence of organic matter ($P<0.001$). Of note was the drop in efficacy of Virkon from approximately 1.2 $\log_{10}$ in the absence of organic matter to approximately 0.2 $\log_{10}$ in the presence of it. Also, interestingly, in the presence of organic matter, for all the agents except Actichlor, the $\log_{10}$ reduction for the epidemic strains was more markedly reduced as compared to that for the non-epidemic strains (Fig. 1). Although for Actichlor, Microsol 3+ and TriGene Advance this difference was not significant, when using Virkon, ribotype 001 was the least effectively destroyed strain ($P<0.05$), and when using Decon 90, strain 630 was the most effectively destroyed ($P<0.001$). As contamination with organic matter is common in the environment, the efficacy of cleaning agents in its presence is of greater practical significance. From the observations above, it is clear that chlorine-based agents are a better choice for disinfection, especially to eliminate the currently epidemic *C. difficile* strains.

Surface decontamination

To test the decontamination of surfaces using the selected agents, 1 cm^2 areas were artificially contaminated with 10^3 spores of each strain, allowed to dry and then cleaned, in an attempt to mimic a real-life situation. Further, washing of the surface was performed by aspiration of disinfectant and subsequent washes. It was observed that only the chlorine-releasing Actichlor was able to completely decontaminate all the surfaces tested; however, it required more than 2 min and up to 10 min to see this effect (Fig. 2). For all the other agents, although there was a greater $\log_{10}$ reduction in spore numbers after 10 min of treatment, it was not significant. Here too it was found that the epidemic strains were less effectively cleaned. Spores of strain 630, which was previously epidemic, were also more resistant to the cleaning, while spores of VPI 10463, which is rarely isolated from patients, were the most effectively destroyed. Thus, though chlorine-releasing agents destroy *C. difficile* spores, the contact times required for surfaces to be decontaminated might be unrealistic due to time constraints as well as the odour, corrosive effects on surfaces and irritation to users (Block, 2004; Ungurs *et al.*, 2011). However, the natural environment might not be as heavily contaminated as the surfaces in these experiments and thus may be more efficiently cleaned. In the laboratory, however, contamination of surfaces by high concentrations of spores is a possibility, and they must be cleaned and decontaminated carefully and thoroughly.

The contaminated environment is important in the transmission of *C. difficile* (Fawley & Wilcox, 2001; Weber *et al.*, 2010), and just as in hospitals, laboratories can be a source of transmission of CDI, due to both the virulent types of strains and the high concentrations of *C. difficile* routinely used. Reports of laboratory-acquired CDI led us to evaluate the type of disinfectants and cleaning agents used in our laboratory in this study. From these experiments, which are preliminary and do not strictly follow the guidelines for disinfectant testing, we can conclude that vegetative cells of *C. difficile* as well as spores can be destroyed by a variety of agents in suspension tests with long contact times. However, hard non-porous surfaces were only decontaminated by the use of a chlorine-releasing agent. Even then, a contact time of up to 10 min was required to eliminate high concentrations of *C. difficile* spores. Thus, chlorine-releasing agents should be the decontaminants of choice, not only in hospitals (Fraise, 2011), but also in laboratories.

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